

# **Biomarkers and targeted therapies for ovarian granulosa cell tumors**

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*To my family*

## Abstract

Adult-type granulosa cell tumor (AGCT) is a unique subtype of ovarian cancer, accounting for 5% of all ovarian malignancies. Due to the hormonal activity and slow growth of the tumor, AGCTs are usually diagnosed at an early stage, and the overall prognosis is favorable; the 5-year overall survival rate is 98%. However, these tumors have a tendency for late relapse, sometimes occurring decades after primary diagnosis. Eventually AGCT recurs in every third patient, and half of them succumb to the disease.

Surgery is the mainstay of treatment, and accurate preoperative diagnosis is crucial for the referral of patients to appropriate surgical units. In most patients no adjuvant therapy is needed after primary operation. Platinum-based chemotherapy is used in advanced and inoperable AGCTs, but its efficacy is very modest, and no targeted therapies exist for AGCT. The objectives of this study were to improve the preoperative diagnostics of AGCTs and to uncover targeted treatments for AGCT.

HE4 and CA125 are commonly used in the preoperative evaluation of unknown ovarian masses, but their roles in AGCTs are unknown. Based on our results, HE4 levels are not elevated in AGCTs. We detected increased CA125 levels in a subset of AGCTs; however, the marker had no prognostic significance. These results underline the possibility of malignant ovarian tumor even in situations where CA125 and HE4 are both normal. Based on our and previous findings, inhibin B and AMH are the most specific markers in distinguishing AGCT from other malignant or benign ovarian tumors.

AGCTs belong to the sex-cord stromal tumors and are thought to be derived from granulosa cells in preantral follicles. Most AGCTs (97%) harbor a point mutation in the gene coding for transcription factor FOXL2. The equilibrium between proliferation and apoptosis is crucial in the physiology of normal granulosa cells and it has been proposed to be disturbed in AGCTs. TNF-related apoptosis inducing ligand (TRAIL) was earlier found to induce apoptosis in AGCT *in vitro*. We evaluated the role of TRAIL in clinical AGCT samples and detected a significant correlation of TRAIL to its receptors (DR4 and DR5) in AGCT tissue samples, indicating active TRAIL signaling in AGCTs. Furthermore, circulating TRAIL was decreased in patients with large AGCTs, implying a tumor suppressive role for TRAIL in AGCTs. This was noted also at the tissue level as decreased TRAIL expression was seen in large AGCTs. Our results suggest a potential role for TRAIL in AGCT treatment.

AGCTs produce estrogens, inhibin and anti-Müllerian hormone, and express various hormone receptors, including receptors for estrogens, androgens and gonadotropins. However, the roles of these hormones in tumor proliferation have remained unclear. We used our vast collection of tissue and serum samples of AGCT patients to characterize the hormonal milieu of AGCTs and studied the functional effects of follicle-stimulating hormone (FSH) and estradiol (E2) in tumor proliferation by using cell culture assays. FSH receptor and estrogen receptor  $\beta$  (ER $\beta$ ) were found to be expressed in the majority of tumors



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whereas the expression of aromatase, a crucial enzyme in estrogen synthesis, varied significantly among the studied tumors. In functional assays, FSH increased the viable cell number in subset of AGCTs, whereas E2 increased the cell number only at high concentrations. The aromatase inhibitor letrozole was able to block E2 synthesis in AGCTs but had no effect on cell viability.

To systematically screen for new targeted therapies for AGCTs, we performed drug sensitivity and resistance testing and transcriptomic profiling. Seven AGCT primary cell cultures were exposed to a collection of 230 oncologic regimens, including both traditional chemotherapeutics, approved targeted drugs and investigational compounds. Among these regimens, a multi tyrosine kinase inhibitor dasatinib emerged as the most effective targeted treatment. Furthermore, dasatinib in combination with the traditional chemotherapeutic paclitaxel showed a synergistic effect in reducing the viability of AGCT cells. RNA sequencing of the screened tumors revealed no mutations in target genes of dasatinib, but these genes were highly expressed. Our results suggest clinical testing of dasatinib together with paclitaxel may be warranted in patients with advanced AGCT.

In conclusion, we verified that inhibin B and AMH are the most specific serum markers in AGCT preoperative diagnostics. Even though aromatase inhibitors showed no efficacy in inhibition of AGCT cell growth, further clinical studies are needed to verify the role of hormonal treatments in AGCTs. Our approach emphasizes the power of hypothesis-based and systematic translational research in finding novel therapeutic targets for ovarian cancer. Importantly, our results suggest a role for TRAIL and dasatinib in AGCT treatment and encourage clinical testing of these regimens.

## Finnish summary

Aikuistyyppin granuloosasolukasvain (AGSK) on munasarjasyövän alatyyppejä, joka käsittää 5% pahanlaatuisista munasarjakasvaimista. Koska kasvain on hormonaalisesti aktiivinen ja hidaskasvuinen, AGSK:t havaitaan yleensä aikaisessa vaiheessa. Taudilla onkin aluksi suotuisa ennuste: 5 vuoden kuluttua elossa on 98% potilaista. Kasvaimella on kuitenkin taipumus myöhäiseen uusiutumiseen, joka voi tapahtua jopa vuosikymmenten kuluttua taudin ensidiagnoosista. Noin kolmasosalla potilaista AGSK uusiutuu, ja näistä noin puolet menehtyy tautiin.

Leikkaus on hoidon kulmakivi. AGSK:t olisi tärkeää erottaa hyvänlaatuisista munasarjakasvaimista jo ennen toimenpidettä, jotta leikkaukset voitaisiin keskittää niihin erikoistuneisiin yksiköihin. Valtaosa AGSK-potilaista ei taudin alkuvaiheessa tarvitse leikkauksen lisäksi muuta hoitoa, ja myös paikalliset uusiutumat pyritään hoitamaan operatiivisesti. Levinnyttä tautia hoidetaan lääkkein, joskin käytössä olevien solunsalpaajien teho AGSK:een on hyvin rajoittunut. Toistaiseksi tähän kasvaimen ei ole olemassa kohdennettuja hoitoja.

Tutkimme kahden munasarjasyöpämerkkiaineen, CA125 ja HE4:n pitoisuuksia AGSK-potilaiden verinäytteissä, ja arvioimme, voiko näitä merkkiaineita käyttää AGSK:n erotusdiagnostiikassa. AGSK-potilailla ei havaittu koholla olevia HE4-tasoja. CA125 oli koholla osalla AGSK-potilaista, mutta tällä ei ollut ennusteellista merkitystä. Normaalit CA125 ja HE4 -tasot eivät poissulje pahanlaatuisen munasarjakasvaimen mahdollisuutta. Tutkimuksemme vahvisti aiemmat havainnot siitä, että inhibiini B ja AMH ovat tarkkoja merkkiaineita erottamaan AGSK:t sekä pahanlaatuisista että hyvänlaatuisista munasarjamuutoksista.

AGSK on lähtöisin sukupienasta, ja sen ajatellaan saavan alkunsa munasarjassa kehittyvän follikkelin granuloosasoluista. Valtaosalla (97%) AGSK-potilaiden kasvaimista löytyy somaattinen mutaatio geenissä, joka koodaa FOXL2-transkriptiotekijää. Vielä ei kuitenkaan tiedetä mutaation tarkkaa vaikutusmekanismia taudin kehittymisessä. Normaaliin granuloosasolujen elinkaarta säätelevät nopeaa kasvua edistävät tekijät, sekä ohjelmoidun solukuoleman mekanismit. Näiden tasapainon on ajateltu olevan AGSK:ssa häiriintynyt. TNF-related apoptosis inducing ligand (TRAIL) on proteiini, jonka on aiemmin todettu aiheuttavan AGSK-soluissa (*in vitro*) ohjelmoitua solukuolemaa. Tutkimme TRAIL-proteiinin roolia AGSK-potilailla, ja osoitimme AGSK-kudoksen TRAIL-ligandin ilmentymisen korreloivan positiivisesti TRAIL-reseptoreiden ilmentymiseen, mikä viittaa aktiiviseen TRAIL-signaalointiin AGSK:ssa. AGSK-potilailla, joilla oli keskimääräistä kookkaammat kasvaimet, TRAIL-proteiinin määrä sekä verenkierrossa että kasvaimessa oli vähentynyt. Tulokset viittaavat TRAIL:n säätelevän AGSK:n kasvua ja puoltavat jatkotutkimuksia TRAIL:ista AGSK-potilaiden hoidossa.

AGSK:t tuottavat hormoneja kuten estrogeenia, inhibiineja ja anti-Müllerian hormonia (AMH). Kasvaimet ilmentävät myös monia hormonireseptoreita, kuten estrogeeni-,

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androgeeni- ja gonadotropiinireseptoreita. Hormonien rooli AGSK:n kehittämisessä ja kasvussa on kuitenkin epäselvä. Tutkimme hormonireseptoreiden ilmentymistä laajassa AGSK-aineistossamme, sekä solumallien avulla FSH:n ja estradiolin vaikutusta solujen kasvuun. FSH-reseptori ja estrogeenireseptori  $\beta$  ilmentyivät valtaosassa AGSK:ssa. Sen sijaan vain noin puolet kasvaimista ilmensi aromataasia, joka on välttämätön entsyymi kudoksen estradiolituoton kannalta. FSH lisäsi AGSK-solujen määrää osassa kasvaimista, kun taas estradioli vaikutti solujen määrää lisäävästi vain hyvin suurilla annoksilla. Aromataasinestäjä letrotsoli esti kasvaimen estradiolituoton, mutta solujen elinvoimaisuuteen sillä ei ollut vaikutusta.

Tutkimuksessa tehtiin myös laaja-alaista lääketestausta AGSK-soluille, tavoitteena löytää uusia täsmällisesti kasvaimen kohdennettuja hoitoja. Seitsemästä AGSK:sta tehtiin soluviljelmä, ja solujen herkkyys testattiin 230 eri lääkeaineelle. Lääkepaneeliin kuului sekä perinteisiä solunsalpaajia, muiden tautien hoitoon jo hyväksytyjä lääkkeitä sekä kokeellisia yhdisteitä. Tyrosiinikinaasistäjä dasatinibi osoittautui tehokkaaksi kaikissa testatuissa kasvaimissa. Kun dasatinibi yhdistettiin paklitakseliin, AGSK:n hoidossa käytettävään solunsalpaajaan, havaittiin näillä lääkkeillä synergiavaikutus. Kasvainnäytteet RNA-sekvensoitiin, ja useiden dasatinibin kohdegeenien havaittiin ilmentyvän AGSK:ssa. Lääkevastetta selittäviä mutaatioita kohdegeeneistä ei löytynyt. Tulosten perusteella dasatinibin ja paklitakselin yhdistelmää kannattaa testata AGSK-potilaiden hoidossa.

Tässä tutkimuksessa vahvistettiin inhibiini B:n ja AMH:n rooli AGSK:n erotusdiagnostiikassa ennen leikkausta. Kohdennettujen hoitojen suhteen aromataasi-inhibiittoreilla ei havaittu olevan solujen kasvua hillitsevää vaikutusta. Osa potilaista saattaa kuitenkin hyötyä hormonaalisista hoidoista. Sekä TRAIL-proteiini että tyrosiinikinaasi-inhibiittori dasatinibi ovat yhdisteitä, joita on aiheellista jatkossa testata kliinisin tutkimuksin AGSK-potilaiden hoidossa.

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## List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

- I            **Haltia UM**, Hallamaa M, Tapper J, Hynninen J, Alfthan H, Kalra B, Ritvos O, Heikinheimo M, Unkila-Kallio L, Perheentupa A, Färkkilä A. Roles of human epididymis protein 4, carbohydrate antigen 125, inhibin B and anti-Müllerian hormone in the differential diagnosis and follow-up of ovarian granulosa cell tumors. *Gynecol Oncol* Jan;144(1):83-89, 2017
  
- II           **Färkkilä A**, Zauli G, **Haltia UM**, Pihlajoki M, Unkila-Kallio L, Secchiero P, Heikinheimo M. Circulating levels of TNF-related apoptosis-inducing ligand are decreased in patients with large adult-type granulosa cell tumors - implications for therapeutic potential. *Tumour Biol* Sep; 37(9):11909-11916, 2016.
  
- III          **Haltia UM**, Pihlajoki M, Andersson N, Mäkinen L, Tapper J, Cervera A, Horlings HM, Turpeinen U, Anttonen M, Butzow R, Unkila-Kallio L, Carpen O, Wilson DB, Heikinheimo M, Färkkilä A. Functional profiling of FSH and estradiol in ovarian granulosa cell tumors. Submitted.
  
- IV          **Haltia UM\***, Andersson N\*, Yadav B\*, Färkkilä A, Kuleskiy E, Kankainen M, Tang J, Butzow R, Riska A, Leminen A, Heikinheimo M, Kallioniemi O, Unkila-Kallio L, Wennerberg K, Aittokallio T, Anttonen M. Systematic drug sensitivity testing reveals synergistic growth inhibition by dasatinib or mTOR inhibitors with paclitaxel in ovarian granulosa cell tumor cells. *Gynecol Oncol* Mar;144(3)621-630, 2017.

\* The authors contributed equally to the study.

## ABBREVIATIONS

### Abbreviations

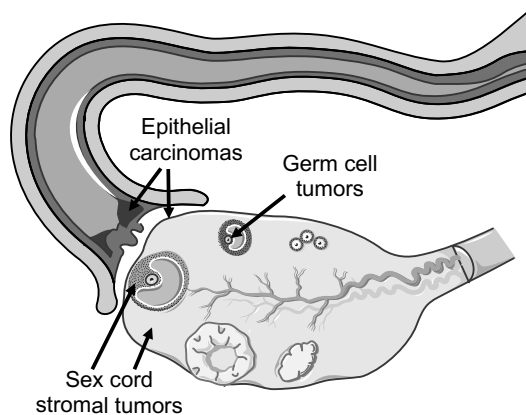
AGCT	Adult-type granulosa cell tumor	3-β-HSD	3-β-hydroxysteroid dehydrogenase
AMH	Anti-Müllerian hormone		
BCL2	B cell lymphoma 2	HE4	Human epididymis secretory protein 4
BEP	Bleomycin-etoposide-cisplatin		
BMP	Bone morphogenetic protein	hGL	Human granulosa luteal
CA125	Cancer antigen 125	IHC	Immunohistochemistry
CCND2	Cyclin D2	KEGG	Kyoto encyclopedia of Genes and Genomes
cDNA	Complementary DNA		
CREB	cAMP-response element binding protein	LC-MS	Liquid chromatography mass spectrometry
CT	Computer tomography	LH	Luteinizing hormone
CYP19A1	Cytochrome P450 19A1 (aromatase)	MRI	Magnetic resonance imaging
DcR	Decoy receptor	mRNA	Messenger RNA
DHEA	Dehydroepiandrosterone	mTOR	Mammalian target of rapamycin
DR	Death receptor	PDGFR	Platelet derived growth factor receptor
DSRT	Drug sensitivity and resistance testing	PI3K	Phosphoinositide 3-kinase
DSS	Drug sensitivity score	PKA	Protein kinase A
E1, E2, E3	Estrone, estradiol, estriol	qPCR	Quantitative polymerase chain reaction
EGF	Epidermal growth factor	RECIST	Response evaluation criteria in solid tumors
ELISA	Enzyme-linked immunosorbent assay	SCST	Sex-cord stromal tumor
ENDO	Endometrioma	SF-1	Steroidogenic factor-1
EOC	Epithelial ovarian carcinoma	SPRI	Solid phase reversible immobilisation
ER	Estrogen receptor	StAR	Steroidogenic acute regulatory protein
FGF	Fibroblast growth factor	TGF-β	Transforming growth factor-β
FOXL2	Forkhead box protein L2	TERT	Telomerase reverse transcriptase
FPKM	Fragment per kilobase of exon per million	TMA	Tissue microarray
FSH	Follicle-stimulating hormone	TNF	Tumor necrosis factor
GATA4	GATA binding protein 4	TPM	Transcripts Per Kilobase Million
GDF	Growth and differentiation factor	TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
GnRH	Gonadotropin-releasing hormone		
GPOR	G-protein-coupled estrogen receptor	WES	Whole exome sequencing



## Review of the literature

### 1 Clinical characteristics of adult type granulosa cell tumors

Ovarian cancer consists of tumors that are histologically and genetically distinct. Three main subtypes exist: epithelial carcinomas (90% of all ovarian malignancies), sex-cord stromal tumors (SCST) (5-8%) and germ-cell tumors (2-3%) (Figure 1) (1, 2). A large proportion of epithelial carcinomas are thought to derive from cells that are not present in the normal ovary, including the high grade serous carcinomas originating from the fallopian tubes and clear cell and endometrioid carcinomas arising from endometriotic lesions. Thus, SCSTs and germ-cell tumors can be regarded as “real intra-ovarian cancers”, as they originate from cells that are located in the normal ovary. SCSTs are derived from either the sex-cord or ovarian stroma, and germ cell tumors originate from the primordial germ cells of the embryonic gonad (reviewed in (3)). Among the SCSTs, granulosa cell tumor (GCT) is the most common subtype, classified as a pure sex-cord tumor (4).



**Figure 1.** Ovarian cancer subtypes have disparate origins. Some epithelial carcinomas originate from the fallopian tube, whereas others are derived from endometriotic lesions. Germ cell tumors arise from the primordial germ cells, and sex cord stromal tumors originate from the ovarian sex-cord or stroma. Adapted from Servier Medical Art ([www.servier.com](http://www.servier.com)).

#### 1.1 Clinical presentation

GCT accounts for 3-5% of ovarian malignancies, with an annual incidence of 0.6-0.8/100,000 (5). In Finland, an average of 22 new cases are diagnosed each year. GCTs are further classified into juvenile and adult subtypes. The juvenile form comprises only 5% of all GCTs and is generally diagnosed in children and adolescents, presenting usually with

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favorable prognosis (6). Adult-type GCTs are usually diagnosed at perimenopause or early postmenopause, although they can occur throughout adulthood (7). This study focuses on adult-type granulosa cell tumors, hereafter referred to as AGCT.

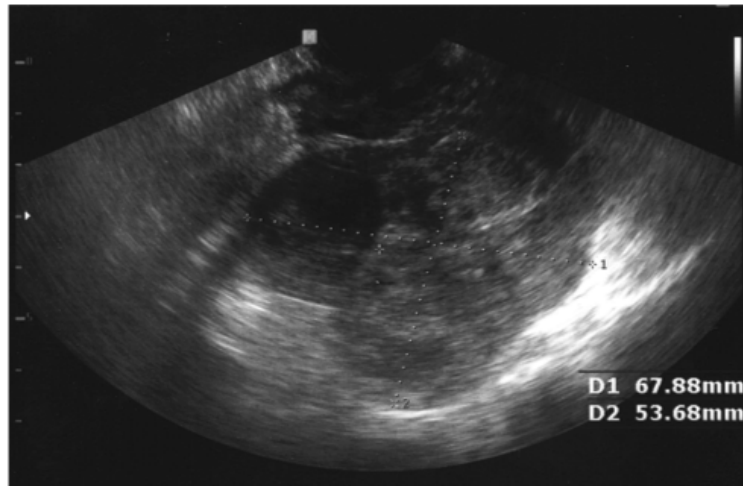
Typically AGCTs are diagnosed at an early stage. This is due to the slow growth and low metastatic potential of these tumors. Furthermore, AGCTs' hormonal activity leads to detectable symptoms early in the course of the disease. At primary diagnosis, 50-80% of the patients have stage Ia disease in which the tumor is inside the ovarian capsule. Approximately 30% of cases are diagnosed at stage Ic, in which tumor cells infiltrate through ovarian capsule or the capsule is ruptured either spontaneously or iatrogenically during the operation (1, 8-10). Advanced stage (stage III-IV) disease at diagnosis is uncommon. The size of the tumor varies; in a largest published cohort from our group the median size was 10.0 cm, ranging from 0.5 cm to 30 cm. Ascites is present in only 23% of the patients (8).

The most typical symptom of AGCT is abnormal uterine bleeding: nearly half of the patients report menstrual disturbances or postmenopausal bleeding at primary diagnosis (8). These symptoms are due to estrogenic activity of AGCT. Patients with large tumors may suffer from abdominal pain and distension. Due to the high vascularization of the tumor, a subset of patients present with acute abdominal pain due to hemoperitoneum caused by tumor rupture (11). Still, more than 10% of the patients remain asymptomatic and tumor is found incidentally in a routine gynecological check-up or radiological imaging (8).

### 1.2 Differential diagnostics

#### *Radiological imaging*

In ultrasound, AGCT typically presents as cystic-solid ovarian mass with high vasculature (12) (Figure 2). Common pattern is either a large solid mass with heterogeneous echogenicity and internal cysts or large multilocular-solid mass without papillary projections (13, 14). These same features are seen in computerized tomography (CT). CT and especially magnetic resonance imaging (MRI) can reveal intracystic hemorrhagic components and enhancement of solid portions or cystic wall (14-16). Patients typically have an abnormally thick endometrium, and pathological examination may reveal endometrial hyperplasia or even carcinoma (26-38% and 6-7% of the patients, respectively) (8, 17). It is noteworthy that fluorodeoxyglucose positron emission tomography (FDG-PET) is not informative in AGCT patients due to poor FDG uptake (18). In conclusion, radiological differentiation of AGCT from other malignant or benign ovarian masses is not definitive and thus complementary diagnostic tools such as serum markers are needed.



**Figure 2.** *Ultrasound image of AGCT.*

### *Serum markers*

In preoperative diagnostics of pelvic tumors, serum tumor markers play a significant role. CA125 has been used as a marker for epithelial ovarian cancer (EOC) for decades. However, there are limitations with CA125 including elevated serum values in benign gynecological conditions, such as in endometriosis and pelvic inflammatory diseases as well as in non-gynecological malignancies (19-21). On the other hand, only half of the early stage EOC patients have aberrant CA125 values (22). Recently, human epididymis secretory protein 4 (HE4) has been accepted for clinical use and particularly in combination with CA125, it presents a sensitive and specific method for preoperative evaluation (23). Unlike CA125, HE4 levels are not affected by peritoneal irritation or endometriosis. Nevertheless, also HE4 has some limitations, as for example smoking, renal insufficiency, and aging may rise the levels in serum (24, 25). Moreover, HE4 is not superior to CA125 in detecting early stage disease (26).

Based on earlier reports, elevated serum CA125 levels have been detected also in some AGCTs (27, 28). So far there are no studies reporting serum HE4 levels in AGCT patients. On the other hand, a number of studies have shown the utility of inhibin B and anti-Müllerian hormone (AMH) in AGCT surveillance (29, 30). Similar to normal granulosa cells in developing follicles, AGCT cells produce these glycoprotein hormones and release them into the circulation (31, 32). One limitation of inhibin B is its known fluctuation during the menstrual cycle. Furthermore, inhibin B levels tend to be elevated in mucinous tumors (33). AMH levels are generally more stable, although the methods for measuring AMH have

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shown inconsistency and thus validation of new methods is required (34). Estradiol has also been suggested as a tumor marker. It can be measured preoperatively as an indication of excess sex steroid production by the tumor (35). According to a previous small patient series the role of estradiol as a tumor marker was uncertain (32, 36).

### *Histopathological diagnosis*

Histologically, AGCTs present with a diversity of patterns, including both well and poorly differentiated appearances, often varying within a single tumor. Well-differentiated types include microfollicular (most common), macrofollicular, trabecular, insular and tubular patterns, whereas the poorly differentiated type is characterized as diffuse (sarcomatoid) pattern (37). Characteristically AGCTs contain round to oval, pale cells with longitudinally grooved “coffee-bean” nuclei and scanty cytoplasm. Call-Exner bodies are typical for the microfollicular type, consisting of small rings of well-differentiated granulosa cells. Mitotic figures are only rarely seen and nuclear atypia is usually mild (37). The histological evaluation of AGCT that is based only on hematoxylin-eosin-staining is challenging and false-positive diagnosis rates up to 36% have been reported (8, 38). Immunohistochemistry for inhibin- $\alpha$  and cytokeratin is clinically recommended and a reticulum stain may differentiate AGCT from theca-derived tumors (39-41). Also calretinin and FOXL2 immunostaining is utilized (42, 43). However, some of the AGCTs remain negative for typical immunohistochemical markers.

### *FOXL2 mutation in diagnostics*

In 2009, a single somatic missense mutation in the gene encoding for transcription factor FOXL2 was identified in AGCTs (44). This point mutation 402C>G(C134W) was subsequently verified in multiple independent cohorts, and according to most studies, it is present in 94-97% of AGCTs (45, 46). *FOXL2* mutation analysis has proven to be invaluable in the diagnosis of AGCT in cases where the histological diagnosis remains uncertain. This was shown recently in a cohort of 336 AGCTs where *FOXL2* mutation negative cases (n=80) were histologically re-evaluated with additional immunohistochemistry (47). The majority of these tumors proved to be originally misdiagnosed. The revised diagnoses included other sex-cord stromal tumors (49.2%), epithelial malignancies (44.4%) and miscellaneous tumors (6.3%) Even though a minor subset of AGCTs with wild type *FOXL2* exists, *FOXL2* mutation analysis can be regarded as a useful tool in the differential diagnosis of AGCTs with challenging histological appearances.

### 1.3 Treatment

#### *Surgery*

The standard treatment for primary AGCT is surgery, consisting of removal of the tumor, uterus, ovaries and fallopian tubes. Surgery also includes staging procedures including peritoneal washings, biopsies from the peritoneal cavity and infracolic omentectomy (48). Suspicious lymph nodes should be removed, whereas routine pelvic and para-aortal lymphadenectomy is not recommended as AGCTs spread pattern is mainly intraperitoneal or hematogenous (48-51). Surgical staging has been reported to be an independent prognostic factor (52). A laparoscopic approach has proven to be a safe option for laparotomy, associated with less morbidity (53, 54). In rare cases with advanced disease, a debulking procedure is recommended. Fertility-sparing surgery may be performed in younger patients with local (stage I) disease, preserving the uterus and normal contralateral ovary. Several older studies report elevated recurrence rates in patients undergone fertility-sparing surgery (55, 56). However, a recent study reported equal disease free and overall survival between conservatively and radically operated patients (57).

Surgery is usually the treatment of choice also in relapsed AGCTs. The most common site for recurrence is the pelvis. Other typical sites for relapses include the abdominal cavity, retroperitoneum, liver and inguinal or para-aortal lymph nodes. Multifocal disease has been observed in close to half of all relapses (58, 59). Altogether, the majority (70-80%) of recurrences can be treated by debulking surgery (58, 60).

#### *Chemotherapy*

Due to the infrequency of AGCT, prospective randomized studies on adjuvant chemotherapy are lacking and treatment recommendations are based on retrospective series with limited number of patients. Generally, adjuvant therapy is recommended after primary surgery in advanced stages (II-IV). In addition, international guidelines suggest consideration of adjuvant chemotherapy for high risk stage I patients, such as stage Ic patients with ruptured tumors or poor differentiation (48, 61). On the other hand, according to multiple reports, adjuvant chemotherapy has no impact on disease-free survival in stage Ic AGCT and one of the studies questioned its efficacy even in advanced disease (52, 57, 62). In recurrences chemotherapy may be used with or without surgery.

In AGCT chemotherapy, platinum-based combinations have been the primary option. For the most commonly used combination of bleomycin, etoposide and cisplatin (BEP), variable response rates from 40-100 % have been reported (63-65). In older studies, patient cohorts have been very small and the definition of response has varied as adequate radiological imaging has only rarely been used. Recently, a study using RECIST (Response Evaluation Criteria in Solid Tumors) criteria, reported either complete or partial response in

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only 22% of the patients (66). The proportion of patients with stable disease was high in this study reaching 78%, but progression was seen after two to five months in some patients. Considering the slow growth tendency of AGCT, these reported responses are very modest. It is also noteworthy that BEP combination has serious adverse effects. Bleomycin causes pulmonary side effects, and bleomycin and etoposide can cause myelotoxicity, associated also with the development of secondary malignancies (67, 68). Taxanes have also been used in AGCT chemotherapy, with putatively less toxic side effects. A retrospective series presented response rates comparable to BEP (69, 70), but in a prospective trial consisting of paclitaxel as a single therapy only modest efficacy was reported (71). Currently there is a randomized phase II trial investigating combination of paclitaxel and carboplatin in treatment of advanced or recurrent SCSTs ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)).

### *Hormonal therapy and targeted treatments*

As AGCTs are known to express hormone receptors, hormonal treatments have been empirically used in AGCTs, especially in cases where the response to chemotherapeutics has been lost. Several agents including progestins, gonadotrophin releasing hormone agonists, selective estrogen receptor modulators and aromatase inhibitors have been utilized with varying responses. The current literature concerning these treatments consists mainly of case reports. A systematic review published in 2014 summarized responses from 19 reports, and a complete or partial response was reported in 22/31 patients (72). Most convincing results were seen with aromatase inhibitors, whereas the selective estrogen receptor modulator tamoxifen showed no effect. However, the selection bias in case report studies is apparent as negative results are rarely reported. In a recent retrospective cohort evaluated by RECIST criteria, response was achieved in only 4/22 (18%) of patients (73). Among patients treated with aromatase inhibitors (anastrozole or letrozole), 7/10 patients had stable disease while disease progression was seen in the remaining three patients.

Bevacizumab, a humanized monoclonal antibody for vascular endothelial growth factor, has become a widely used targeted therapeutic in epithelial ovarian cancer (74). It has also reported to have activity in sex-cord stromal tumors. Of 36 patients enrolled in a phase 2 trial, six patients (17%) had partial response, while 78% had stable disease. (75). Recently, in a randomized clinical study bevacizumab was used together with weekly paclitaxel in relapsed sex-cord stromal tumors, of which majority (87%) consisted of AGCTs (76). Even though the objective response rate was superior in the treatment group at six months (44.4% and 25.0% in treatment and control group, respectively), addition of bevacizumab to paclitaxel did not result in increased progression free survival. Altogether these responses are only modest and do not support the use of bevacizumab in routine AGCT therapy.

### 1.4 Follow-up and recurrence

Follow-up of AGCT patients consists of regular gynecologic examination, transvaginal ultrasound and serum tumor markers, specifically inhibin B and AMH. Markers are crucial especially in detecting relapses outside pelvis. Increased inhibin B levels have been reported 5-20 months prior to clinical onset of the disease, suggesting it may serve as an early detection marker (30). A corresponding time scale of 11 months has been reported for AMH (77). There is strong evidence for both inhibin B and AMH in AGCT follow-up, and these markers are considered equally sensitive (93 and 92% for inhibin B and AMH, respectively) and specific (83% and 82%) (29). The combination of inhibin B and AMH has proven to be even more accurate in detecting macroscopic disease. In our recent study, circulating tumor DNA was detected in patients with macroscopic tumor, and this method may thus offer an option for AGCT surveillance in the future (78). In the case of suspected recurrence based on serum markers or clinical findings, CT scan is recommended. Guidelines from the European Society for Medical Oncology recommend follow-up at three-month intervals during first two years, followed by six-month intervals until five years (48). The National Comprehensive Cancer Network guidelines recommend lengthening follow-up beyond five years (61).

AGCT is characterized by slow and indolent growth. However, a unique feature of this tumor is its tendency for late relapse, reported even 41 years after primary diagnosis (79). Recurrence rates in the latest reports have varied between 20-30% (58, 80, 81). In our *FOXL2*-mutation validated patient cohort with a mean follow-up-time of 16.8 years, the recurrence rate was 32.1% (58). In this patient cohort, the median time from primary diagnosis to the first recurrence was 7.4 years (range 1.1-26.6), supporting the extension of follow-up beyond five years. Due to an active surgical approach, a significant portion of patients attain complete response also after treatment of recurrence; however, successive relapses are common and even up to six recurrences have been reported in patients. Disease-free intervals shorten progressively: in our cohort the median time from first to second relapse was 2.9 years, (range 0.7-29.6) and the time from second to third relapse 1.4 years (range 0.7-4.4).

### 1.5 Prognosis and survival

Various clinical factors have been investigated for their prognostic value in AGCT, but so far tumor stage remains the only identified prognostic indicator (8, 10, 60). Among stage I patients, stage Ic patients (with tumor infiltrating ovarian capsule or tumor rupture intraoperatively) possess a significantly increased risk for recurrence (58, 81). Also multiple histopathological factors have been evaluated in regards to AGCT prognosis, from which high-level nuclear atypia and high expression level of transcription factor GATA4 have shown prognostic value (82). Nevertheless, none of the markers has been validated for clinical use.

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The 5- and 10-year survivals in AGCT have significantly varied among cohorts, from 77% to 94 % (5-year survival) and from 67% to 87% (10-year survival) (8, 47, 64). However, older cohorts often contain misdiagnosed tumors affecting the survival statistics. In our *FOXL2*-mutation validated cohort, disease-specific 5- and 10-year survival was 97% and 92% respectively, and the overall survival reached 84% at ten years. Even though these rates are excellent, 50-80% of the patients with relapsed disease eventually die from AGCT (8, 38, 47).



## 2 Physiology of normal granulosa cells

### 2.1 Folliculogenesis

Human ovary consists of three distinct regions: an outer cortex including the germinal epithelium and follicles, a medulla containing the stroma and a hilum where the ovary is attached to the mesovarium. During the reproductive lifespan of a woman, ovarian follicles are responsible for producing the sex hormones and for supporting the ova to become fertilized.

Follicle development starts during the fetal period. At birth approximately one million primordial follicles reside in the ovary, each containing a primary oocyte. The number of primordial follicles decreases rapidly, and at the onset of puberty only 300 000-400 000 still exist. During female reproductive years, approximately 300-400 primordial follicles finish the maturation process leading to ovulation and formation of corpus luteum. This process is called folliculogenesis (reviewed in (83)).

#### *Initial recruitment*

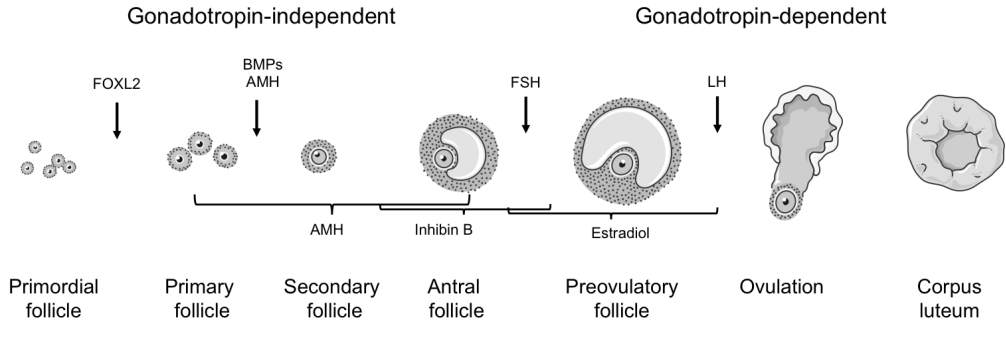
Follicles consist of granulosa cells (GC), which surround the oocyte and provide physical support and the required microenvironment for its maturation. In primordial follicles GCs are small, flat and quiescent, and form only a single layer. During the initial recruitment GCs transform from squamous to cuboidal and grow in size as more cell layers are produced (83). The outer layer surrounding the follicle, theca, is formed at the secondary stage. This initial growth of primordial and primary follicles is hormone-independent and steered mainly by the oocyte and by granulosa cell expressed transcription factors and paracrine signalling. Of the granulosa cell expressed transcription factors Forkhead box L2 (FOXL2) is the most well-known, and it has been regarded as the female sex-determining factor (84). FOXL2 is considered crucial in the development of follicles from primordial to primary state regulating the transition of GCs from squamous to cuboidal (85). In addition to granulosa cells, FOXL2 is present in a developing eyelid and it was initially recognized as mutated in blepharophimosis-ptosis-epicanthus inversus syndrome with primary ovarian failure in patients (86). At primary state follicles start to express TGFβ-superfamily members such as AMH and bone morphogenetic proteins (BMPs). Most BMPs stimulate follicle growth, whereas AMH is thought to inhibit the initial recruitment of primordial follicles (87, 88).

#### *Cyclic recruitment*

As the initial recruitment happens from the fetal period until menopause, cyclic recruitment begins at puberty eventually leading to menstrual cycles (83). In each cycle only a few

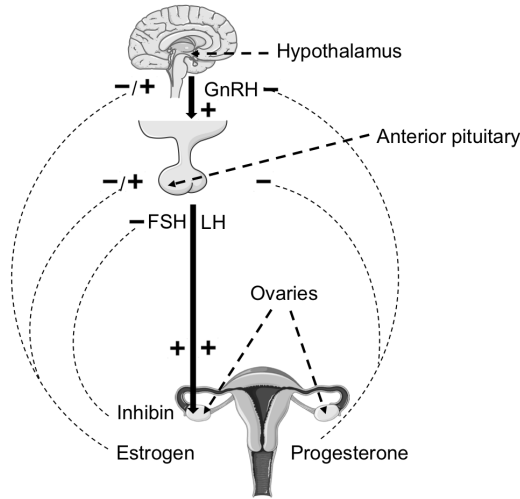
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follicles are recruited and thus rescued from atresia. In recruited follicles granulosa cells proliferate rapidly, and a fluid-filled space called antrum is formed. At this antral stage, granulosa cells can be divided into mural and cumulus cell types reflecting their location in the follicle. Usually only one follicle is chosen to become dominant, leading to further rapid growth, ovulation and eventual fertilization. Meanwhile, all the other recruited follicles proceed to atresia. The various follicle types are shown in Figure 3.



**Figure 3.** Folliculogenesis. Factors contributing to folliculogenesis are shown above the follicles, and hormones secreted by follicles are shown below. Adapted from Servier Medical Art ([www.servier.com](http://www.servier.com)).

Follicle growth during initial recruitment is controlled by autocrine and paracrine factors, but during cyclic recruitment endocrine regulation becomes important (83). From the pre-antral stage forwards follicular cells express receptors for pituitary secreted gonadotropins follicle-stimulating-hormone (FSH, in granulosa cells) and luteinizing hormone (LH, in theca cells). The hypothalamic-pituitary-gonadal axis is thus established (Figure 4). From puberty onwards, secretion of FSH and LH is regulated by pulsatile release of gonadotropin-releasing hormone (GnRH) by hypothalamus. Granulosa cells respond to FSH stimulation by progressing and secreting hormones such as estradiol and the inhibins (A and B). Inhibin B further regulates FSH secretion by a negative feedback-loop to pituitary.



**Figure 4.** Hypothalamic-pituitary-gonadal axis. Adapted from Servier Medical Art ([www.servier.com](http://www.servier.com)).

## 2.2 FSH

FSH controls proliferation and steroidogenesis in GCs (83). FSH receptor (FSHR) is a G-protein coupled receptor located in the cell membrane. FSHR is activated by binding of the ligand, leading to activation of the classical adenylyl cyclase/cAMP/protein kinase A (PKA) signaling pathway. Signal transduction results in the phosphorylation of the transcription factor cAMP-response element binding protein (CREB) which regulates genes integral for cellular proliferation including CYP19A1, cell cycle regulator cyclin D2 and inhibin  $\alpha$  and  $\beta$  (89). In addition to PKA signaling, FSH exerts its effect via other cellular pathways such as PI3K/Akt and ERK and MAPK (90, 91). For the proper FSH response interplay with insulin-like growth factors (IGF-1 and IGF-2) and estradiol is needed (92, 93).

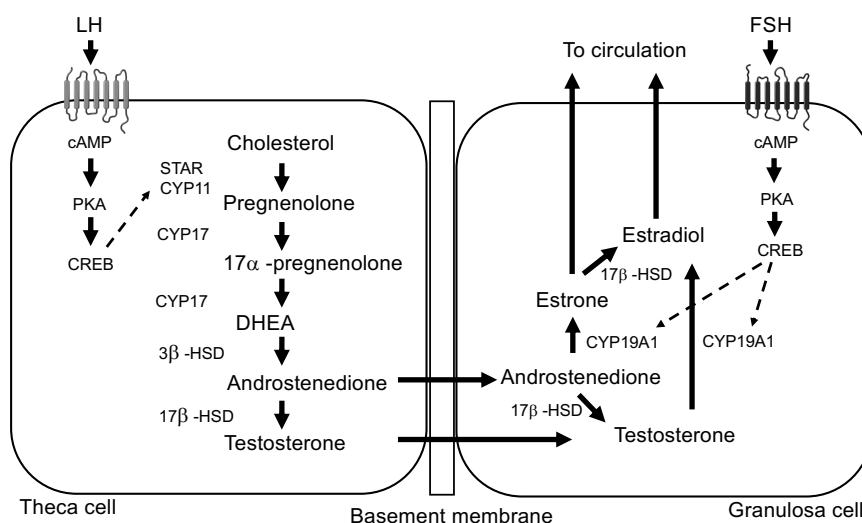
## 2.3 Estrogen synthesis

In addition to supporting the maturation of oocytes, ovarian follicles are responsible for the sex steroid production. These roles are intertwined, as hormonal interplay is required for the competent maturation process of a gamete. In premenopausal woman, estrogen is principally produced by ovaries, but smaller amounts are synthesized from steroid precursors in nongonadal organs, including the skin (adipose tissue), liver, brain and heart

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(94). Three major forms of estrogens exist: estrone (E1), estradiol (E2, 17 $\beta$ -estradiol) and estriol (E3). E2 is the most potent estrogen in premenopausal women, whereas E3 is the weakest and formed mainly by placenta during pregnancy. E1 is produced mostly by adipose tissue and is the primary estrogen in postmenopausal women (95). Ovary-derived estrogens have endocrine actions as they are secreted into the circulation and target estrogen-responsive tissues, whereas estrogens from non-gonadal tissue act mainly by autocrine and paracrine signaling maintaining tissue-specific functions (96).

The cells taking part in ovarian estrogen synthesis include theca cells (production of androgens from cholesterol) and mural granulosa cells (production of estradiol from androgens) (Figure 5) (97). Conversion of cholesterol into androstenedione requires a five-step process. First, cholesterol is transported from the cytosol of theca cell to mitochondrial inner membrane by the steroidogenic acute regulatory protein (StAR) (98). This is followed by consecutive actions of three enzymes: cholesterol side-chain cleavage (Cyp11A1), 17 $\alpha$ -hydroxylase (Cyp17A1) and 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD). The end product of this chain of reactions is androstenedione, which is secreted and delivered through the basement membrane to adjacent mural granulosa cell (97). Mural granulosa cells are responsible for the conversion of androstenedione to estradiol. For this, the subsequent action of two enzymes is needed: aromatase (Cyp19A1) converts androstenedione to estrone, and 17 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD) dehydrogenates estrone to estradiol.



**Figure 5.** Estrogen synthesis in theca and granulosa cells of the ovary. Gonadotropins FSH and LH regulate steroidogenesis via PKA signaling. Several enzymes are needed in this process.

### *Regulation of steroidogenesis*

The aforementioned process of steroidogenesis is referred as the “two cell, two gonadotropins” theory, referring to theca and granulosa cells and their regulation by two pituitary gonadotropins: FSH and LH (97). Structurally FSH and LH are dimeric glycoproteins with a common  $\alpha$  subunit and diverge from each other by a different  $\beta$  subunit. Theca cells express receptors for LH (LHR) and granulosa cells express receptors for FSH (FSHR).

FSHR and LHR belong to the group of G-protein-linked receptors known for their seven transmembrane  $\alpha$ -helix structure (99). The extracellular domain of the receptors is glycosylated and is capable of binding the hormone. The intracellular part of the receptor is rich for serine and threonine residues which enable the phosphorylation and further activation of PKA/cAMP pathway. This signaling proceeds through activation of CREB and results in expression of FSH/LH responsive genes. Besides CREB, other transcription factors, such as Steroidogenic factor 1 (SF1), SMADs, FOXL2 and GATA factors, take part in regulating gene expression by forming complexes in gene promoters. FOXL2 is capable of suppressing steroidogenic proteins StAR and CYP19A1 by transcriptional repression (100, 101). It also binds SF1 (102).

One of the main gene targets of FSH is *CYP19A1*, a gene encoding aromatase, which catalyzes the rate limiting step in estrogen synthesis (103). Aromatase belongs to the cytochrome P450 superfamily, and besides the gonads, it is expressed in many other tissues such as in the adipose tissue and brain (104, 105). *CYP19A1* gene contains a regulatory region with tissue-specific promoters and also alternative splicing and posttranslational modifications influence on its expression in various tissues. In the ovarian follicles CYP19A1 is localized to mural granulosa cells (106). In addition to FSH, several compounds modulate CYP19A1 activity: TGF $\beta$ , E2, insulin growth factor-1 and androgens enhance the effects of FSH whereas inhibin and BMP-15 among others inhibit the stimulatory effect (107-109).

### **2.4 Estrogen receptors**

In addition to producing estrogens, granulosa cells express estrogen receptors and are regulated by estradiol. Estrogens regulate multiple physiological processes such as cell growth and differentiation. To date, three main estrogen receptors are recognized: nuclear receptors ER $\alpha$  and ER $\beta$  and a cell-membrane localized G-protein coupled estrogen receptor-1 (GPER1). Receptors are distributed in various organs and tissues, including ovaries, bone, breast, heart and brain (reviewed in (110, 111)).

ER $\alpha$  and ER $\beta$  are nuclear transcription factors that are activated by ligands and regulate multiple target genes. Even though they act in the nucleus, these estrogen receptors can also be found in the cytoplasm and mitochondria. Despite their structural similarities, they are

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encoded by different genes and show distinct functional patterns, often counteracting each other. They differ from each other by ligand recognition, recruitment of co-regulatory factors and their target genes. Also, their distribution in tissues varies. In the ovary, ER $\alpha$  is localized in the ovarian surface epithelium, thecal cells and stroma, while ER $\beta$  is the dominant receptor in the granulosa cells (112).

Whereas the transcriptional processes mediated by nuclear ERs are usually slow lasting hours or days, the membrane-based GPER1 mediates rapid actions by activating intracellular signaling cascades. In the ovary, GPER1 is expressed most intensely in the ovarian surface epithelium and theca cells, while granulosa cells exhibit weaker staining pattern (113). According to mouse model studies, loss of ER $\alpha$  has resulted in complete infertility and gonadal deficiencies, whereas loss of ER $\beta$  has caused milder perturbations (114-116). Interestingly, in GPER1 knock-out mouse models no effects on reproduction were noted but changes in metabolism and other physiological processes were seen (117, 118). These results underline the significance of traditional nuclear receptors ER $\alpha$  and ER $\beta$  in ovarian physiology.

### 2.5 Androgen receptors

Androgen action is also important in ovarian function. Nuclear androgen receptors are expressed in granulosa cells of developing follicles, from the primordial stage onwards, increasing gradually and peaking in the small preantral follicles (reviewed in (119)). Androgens are necessary precursors for estrogen synthesis, but they also contribute to the growth of follicles by increasing GC proliferation. They have been shown to enhance the FSHR expression and decrease the expression of growth-inhibitory AMH in preantral follicles. The principal mode of androgen action is via regulation of gene transcription in the nucleus, but recent studies have shown that androgens act via other mechanisms, such as activating growth receptors and signaling pathways (120).

### 2.6 TGF $\beta$ -superfamily

The transforming growth factor  $\beta$ -superfamily consists of more than 35 members, including the inhibin-activin system, various bone-morphogenetic proteins and AMH (reviewed in (121)). TGF $\beta$ -superfamily ligands mediate their biological effects in a cell by phosphorylation of receptor serine-threonine kinases and intracellular Smad proteins, which transmit the signal to nucleus and regulate gene expression. In growing follicles these ligands contribute to bidirectional communication both between GCs and the oocyte and between GCs and theca cells. In addition to paracrine and autocrine actions, these ligands have endocrine functions.

Inhibins and activins are dimeric proteins, consisting of two monomeric protein subunits ( $\alpha$ ,  $\beta$ A or  $\beta$ B). Different combinations of these three protein subunits result in dimeric

proteins designated either as inhibins or activins. Inhibin A consists of  $\alpha$  and  $\beta A$  -subunits, whereas inhibin B is formed when  $\alpha$  and  $\beta B$  -subunits dimerize. During follicle maturation activin promotes the proliferation of granulosa cells and the production of aromatase, while inhibin opposes these effects by decreasing follicle growth (122). The effects of inhibin are mediated by antagonizing activin binding and signaling and also via endocrine action by decreasing pituitary FSH secretion (123-125).

AMH expression is detected in follicles from the primary stage through to the small antral stage. It has an inhibitory role on primordial follicle recruitment, and it also attenuates the FSH-responsiveness of growing follicles (126). On the other hand, in some studies FSH has been shown to stimulate AMH expression in GCs, although there is contradictory data (127, 128). AMH secreted by GCs can be measured in serum and used as a marker of ovarian follicle reserve reflecting the reproductive capacity of a woman (129).

### 2.7 Apoptosis

In each menstrual cycle approximately 1000 follicles start growing and typically only one is further selected, while all the other developing follicles go into atresia (97). Cells in atretic follicles undergo apoptosis, a programmed cell death. This is a highly regulated physiological process existing in multicellular organisms and essential for proper function of tissues. Apoptosis leads to formation of apoptotic bodies, which are later phagocytosed by neighboring cells and macrophages, without generating an inflammatory response. An equilibrium between proliferation and apoptosis of granulosa cells is thus fundamental in folliculogenesis. Ovarian apoptosis is regulated by gonadotropins and growth factors, including IGF-1, EGF and FGF (130, 131). Regarding sex hormones, estradiol has been shown to inhibit granulosa cell apoptosis, and androgens seem to antagonize this effect (132).

Generally, apoptosis is regulated by two distinct routes: extrinsic and intrinsic pathways. The extrinsic pathway is activated by extracellular ligands that bind to death receptors (DR) situated in the cell membrane. The intrinsic pathway is activated by intracellular signaling from mitochondria mainly caused by severe cellular stress including hypoxia and DNA damage. The pathways are intertwined and both of them activate caspases, which cause protein degradation (reviewed in (133)).

The extrinsic pathway is mostly controlled by members of the tumor necrosis superfamily. The best characterized ligand-receptor complexes include FasL/FasR, TNF $\alpha$ /TNFR1, TRAIL/DR4 and TRAIL/DR and all these complexes have shown to be active in the ovary (134-136). TRAIL, also called as Apo2L, is a transmembrane glycoprotein consisting of 281 amino acids, and its extracellular domain can actively be cleaved from the cell membrane to form a soluble cytokine (137). The apoptotic effects of TRAIL are transduced via its receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) which can further activate caspases (138, 139). TRAIL can also bind to decoy receptors DcR1 (TRAIL-

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R3) and DcR2 (TRAIL-R4), which lack the cytoplasmic death domain and thus cannot transduce apoptosis (140, 141), but modulate TRAIL pathway activity by competing the ligand-binding with DR4 and DR5 (142). Most normal tissues including adult ovaries, express TRAIL (143, 144). Additionally TRAIL receptors DR5, DcR1 and DcR2 are expressed in both oocytes and granulosa cells of primary and secondary follicles (145). In porcine granulosa cells TRAIL has been shown to induce apoptosis whereas removal of decoy receptor DcR1 from the cells increased the number of apoptotic cells, suggesting the role of TRAIL pathway in the granulosa cell apoptosis (146).

The intrinsic pathway is mostly regulated by Bcl-2 family, which include both proapoptotic or antiapoptotic members. Also survivin, a member of the inhibitor of apoptosis protein family, decreases intrinsic apoptosis by inhibiting caspases and controlling the cell cycle (147, 148). Survivin, encoded by the *BIRC5* gene, is present during the fetal period but is not detectable in most differentiated tissues. However, there is evidence of survivin expression in hen granulosa cells, with expression levels diminishing over the course of follicle growth and differentiation (149). Expression levels were also dependent on the phase of mitosis. These data suggest a role for survivin in granulosa cells, both in cell cycle regulation and inhibition of apoptosis.



### 3 Pathogenesis of AGCTs

AGCTs share common features with granulosa cells in preantral follicles (150). The gene expression pattern is similar to these rapidly proliferating granulosa cells, and both cell types are capable of producing estrogen and inhibin. Although the biology of granulosa cells is well known, the molecular alterations in AGCT are not.

Unlike many other ovarian malignancies, AGCTs have a relatively stable karyotype. The most frequent chromosomal aberrations entail gain of chromosomes 12 and 14 and loss of chromosome 22, reported in 30-53% of AGCTs (151, 152). No common recurrent driver mutations have been recognized, and even the commonly mutated tumor suppressor gene p53 is intact in AGCTs (153, 154). Transcriptomic studies comparing gene expression in AGCTs and normal granulosa cells have reported increased expression of genes involved in cell proliferation whereas genes linked to cell death show decreased expression (155). Currently the only undisputed recurrent genetic feature for AGCT is the mutation in transcription factor *FOXL2*.

#### 3.1 *FOXL2*-mutation

In 2009, Shah et al performed whole transcriptome RNA sequencing of four AGCTs and identified a somatic missense mutation (402C>G) in a gene encoding for transcription factor *FOXL2* (44). At protein level this mutation leads to substitution of a tryptophan residue for a highly conserved cysteine residue at amino acid position 134 (C134W). This specific mutation has been confirmed to be present in 94-97% of AGCTs (45, 47). This, coupled with its absence in other tumors, implies it is pathognomonic for AGCT (156).

Since the discovery of the *FOXL2* mutation, multiple studies have been conducted to clarify the role of the mutant protein in AGCT pathogenesis. *FOXL2* protein structure is not significantly changed, but alterations in posttranslational modifications, such as phosphorylation and ubiquitination, have been reported (157-159). This may lead to changes in protein-protein interactions and thus affect transcription of target genes (160). When differentially expressed genes between wild type (wt)-*FOXL2* and mutant *FOXL2* were studied, there were abundance of genes for functional annotations of tumorigenesis, cell proliferation and cell death (161). *FOXL2* is normally a negative regulator of cell cycle progression and upregulates genes involved in apoptosis, whereas the mutated *FOXL2* increases progression and attenuates apoptosis. Genes regulated by mutated *FOXL2* have also been shown to be enriched in TGF $\beta$  pathway members, including *SMAD* family members 3 and 6, inhibin  $\beta$ A and receptors for activin. Another study has shown an inhibitory effect of mutant *FOXL2* on follistatin, another member of TGF $\beta$ -family, normally having antiproliferative effects (162). Even though wt-*FOXL2* is considered to abate proliferation and promote apoptosis, data concerning its role as a tumor suppressor gene are still conflicting.

### 3.2 Other genetic alterations

Recently, a few studies have shed more light on the mutational landscapes of AGCTs by using whole exome sequencing (WES) of both primary and recurrent tumors. First, a C228T promoter mutation in telomerase reverse transcriptase (*TERT*) was identified in subset of AGCTs resulting in significantly longer telomeres compared with mutation negative tumors (163). *TERT* is the catalytic subunit in telomerase, and it is usually silenced in differentiated cells, while in cancer cells it may be activated and thus act as tumorigenic factor (164, 165). Interestingly, mutation positive patients (22% of primary tumors and 41% of recurrent tumors) had worse overall survival when compared with mutation negative patients, indicating a role for *TERT* in AGCT progression. Another study using WES data from 22 AGCTs suggested an association of AGCT pathogenesis with DNA repair and EGFR family pathways, but failed to identify any molecular alterations linked to recurrence or aggressiveness of tumors (151). However, in another WES based study a truncating mutation in the histone lysine methyltransferase gene *KMT2D* was found to be present in 3% and 23% of primary and recurrent AGCTs, respectively (166). *KMT2D* has earlier been identified as a tumor suppressor gene in selected lymphomas (167) and the reported association with its inactivation and AGCT recurrence raises interest towards its role in AGCT pathogenesis.

### 3.3 Hormonal activity

The role of FSH-pathway has long been discussed in AGCT pathogenesis. Functional FSH receptor has been reported to be expressed in AGCTs; however, no mutations in the *FSHR* gene have been detected (168-170). A unique feature of AGCTs is the ability of the tumor to secrete inhibin, which further acts by depressing pro-growth signals (36). Pituitary FSH-production is also strongly suppressed by inhibin B, suggesting FSH-independent growth of AGCTs.

FSH induces *CYP19A1* in normal granulosa cells, but studies characterizing *CYP19A1* in AGCTs are scarce. In one study, *CYP19A1* expression was seen in 60% of AGCTs, while in another study it was observed in nearly all tumors (171, 172). This latter study proposed that estrogenicity of AGCTs was dependent on the presence of androgen producing cells in the tumor. Another study identified the *CYP19A1* promoter as a direct target of FOXL2, and suggested that mutated FOXL2 activates *CYP19A1* more than wt-FOXL2 (173). Activity of *CYP19A1* in AGCTs can be inferred since most of these tumors produce estrogen.

Considering the significance of estrogen signaling in normal granulosa cells and the role of estradiol in initiation and progression of several endocrine cancers, tumor-derived estradiol has been proposed to have a role in AGCT pathogenesis (reviewed in (174)). In other hormone-related cancers like breast cancer, the proliferative effects are mainly mediated by ER $\alpha$  receptor while ER $\beta$  seem to oppose these effects and act more as a tumor suppressor. Regarding both ER $\alpha$  and ER $\beta$ , truncating variants have been detected in

cancerous tissues. Whereas the wild-type ER $\beta$  associates with improved survival in breast cancer, truncating variant ER $\beta$ 2 is correlated with a worse prognosis and metastatic disease. In AGCTs, ER $\beta$  has been shown to be the dominant receptor expressed in virtually all AGCTs, whereas ER $\alpha$  is expressed in approximately 30% (175). A recent study concerning AGCTs reported expression of ER $\beta$ 2 in mitochondria of AGCT cells and further interactions with a proapoptotic Bcl-2 family member, thus implicating ER $\beta$ 2 as an antiapoptotic agent in AGCTs (175). It has also been reported that repression of the NFkappaB pathway inhibits the normal actions of estradiol bound to nuclear estrogen receptors via Smad3 (176, 177).

Similar to ER $\beta$ , expression of the progesterone receptor (PR) and androgen receptor (AR) have been shown to be abundant in AGCTs (178-180). Even though progestins have occasionally been used in AGCT treatment, no further studies on their role in AGCT exists (72). Neither have any studies concerning androgenic activity in AGCTs been performed.

Thus far, a couple of groups have studied the effects of estradiol stimulation in AGCT cell line KGN. Nevertheless, no effect on cell viability has been recorded (175, 181). Interestingly, when effects of E2 were studied in a migration assay, E2 could prevent the metastatic spread of AGCTs. This was thought to be mediated by a nongenomic mechanism involving the membrane receptor GPER (181).

### 3.4 TGF $\beta$ -pathway

Many members of TGF $\beta$ -superfamily have an active role in folliculogenesis and on the other hand they are also linked to tumorigenesis in many different cancer types. Multiple studies confirm its role in AGCT and link mutated FOXL2 to various members of this pathway (161, 162). TGF $\beta$ -superfamily ligands act by binding to transmembrane type I or II receptors, further activating the intracellular SMAD proteins. Of the ligands, activin has been shown to promote AGCT formation by increasing SMAD2/3 levels whereas AMH, another ligand of TGF $\beta$ -superfamily, is linked to SMAD 1/5 activation thus inducing apoptosis and inhibiting AGCT growth (182, 183). Also inhibin  $\beta$  is proposed to function as an antagonist of activin and BMP signaling, serving as a growth restricting factor (124, 184).

A physical interaction between FOXL2 and the TGF $\beta$ -pathway signal mediator SMAD3 has been documented in AGCTs (185). SMAD3 has been shown to promote AGCT survival through activation of transcription factor NF-kappaB and appears as an important regulator of cyclin D2 (CCND2), essential in cell cycle and proliferation (186). Together with FOXL2, SMAD3 also regulates gonadotrophin releasing hormone promoter interfering with the hormonal modulation of AGCTs (157). In individual AGCTs SMAD3 mutations have been reported, but their pathogenetic impact is not yet determined (151).

### 3.5 Apoptotic pathways

Dysregulation of apoptosis is one of the hallmarks in cancer evolution. The different apoptotic mechanisms have previously been presented (chapter 2.6). As apoptotic pathways are also essential in normal granulosa cells, disturbances in apoptosis are considered crucial in development of AGCT.

It has previously been reported, that overexpression of FOXL2 induces apoptosis of rat ovarian granulosa cells (187). By using the AGCT cell line KGN, the C134W mutated FOXL2 was shown to display minimal cell death compared with the wild type FOXL2 (188). This was due to differences in caspase activation and differences in regulation of death receptors, such as receptors for TNF $\alpha$  and Fas-ligand.

Indeed, the interplay between various transcription factors seems to be fundamental in AGCT biology. In addition to FOXL2 and SMAD3, a zinc finger transcription factor GATA4 is essential in AGCT pathogenesis and all these three transcription factors have been shown to interact and modulate AGCT cell viability and apoptosis together (185). GATA4 is upregulated by FSH and TGF $\beta$  and involved in proliferation of normal granulosa cells by regulating AMH, inhibin- $\alpha$  and CYP19A1 (189-191). In AGCTs GATA4 correlates positively with proliferation promoting cyclin D2 and apoptosis inhibitor Bcl-2 (192). In functional studies with AGCT cells, overexpression of GATA4 has been noted to protect AGCT cells from apoptosis (193). High GATA4 expression is also considered a prognostic marker as it correlates positively with increased risk of recurrence and shorter disease specific survival (82).

There is convincing data on the role of TNF superfamily member TRAIL in AGCTs. Generally, TRAIL is considered as a promising anticancer agent, due to its ability to induce apoptosis primarily in transformed cells while not affecting normal cells (194). This is believed to be mediated by distinct expression of TRAIL receptors in malignant and normal cells (195). TRAIL was capable of inducing apoptosis in AGCT cell line KGN and primary AGCT cell cultures, while the TRAIL induced apoptosis was inhibited by GATA4. TRAIL receptors DR4 and DR5 were abundantly expressed in AGCTs, confirming functionality of TRAIL pathway in AGCTs and raising interest in TRAIL as a potential AGCT treatment (136, 196).

## 4 Precision medicine approach

The mechanism of action of conventional chemotherapy is based mostly on cytotoxicity. Chemotherapeutics affect the cell cycle and cell proliferation by for example interfering with DNA synthesis and replication, however their mechanism of action makes no difference between malignant and rapidly dividing benign cells. This causes multiple adverse effects: the most common problems include myelosuppression, gastrointestinal problems and neuropathy (197-199). Besides the side effects, another important limitation for the use of chemotherapeutics is the ability of cancer cells to develop drug resistance (200).

As genomic knowledge increases and pathogenetic mechanisms of various cancers are elucidated, the principle of medication is shifting towards approaches targeting specifically cancer cells. The aim of precision medicine is to tailor treatment decisions according to genomic and proteomic features of a patient's tumor (201). Besides aiming at maximal treatment benefit of each individual patient, personalized medicine strives to reduce adverse treatment effects, as therapies are directed at mechanisms prevailing especially in cancer cells. Even the classification of tumors is changing: whereas earlier the tumors were classified according to their location or anatomic origin, nowadays they can be reclassified based on their genomic background (202). A crucial aspect in precision medicine is the necessity of prognostic and predictive biomarkers. Prognostic markers are needed for example to identify patients who are likely to experience a particular outcome, whereas predictive markers are crucial in assessing patients' probable response for a drug in question (203). This also produces economical sustainability, as expensive targeted treatments can be avoided in patient groups without any proven benefit for a certain therapy.

However, major challenges are still to be confronted. Most cancers show considerable intertumoral heterogeneity, arising from mutational variability and alternating epigenetic regulation in tumors between different individuals. In addition to these intertumoral changes, significant genetic variation can also be detected intratumorally (204, 205). Especially in tumors harboring oncogenic driver mutations, multiple distinct cell clones can be present. Intratumoral heterogeneity is one of the reasons leading to acquired resistance of chemotherapeutics (206).

### 4.1 Targeted treatments in clinical use

Hormone therapies were one of the first targeted treatments in clinical use, and have been mainstays of breast and prostate cancer treatment for several decades (207, 208). In 1990s, the first therapeutic monoclonal antibodies rituximab and trastuzumab targeting lymphoma and breast cancer cells were produced. Since then, growth signal inhibitors, apoptosis inducers and angiogenesis inhibitors have been developed for clinical use. These compounds include both small molecular compounds penetrating the cell membrane and monoclonal antibodies binding to cell surface antigens (209).

Growth signal inhibitors typically consist of small molecular regimen inhibiting intracellular kinases. One of the first approved targeted drugs has been imatinib, targeting BCR-Abl tyrosine kinase in chronic myeloid leukemia patients overexpressing BCR-Abl (210). Since approval of imatinib, multiple tyrosine kinase inhibitors have emerged and are currently in clinical use. The first apoptotic modulator for clinical use, Bcl-2 inhibitor venetoclax, was approved in 2016 and is currently used for treating lymphocytic lymphoma and leukemia (211). Angiogenesis inhibitors impair tumor vessel formation leading to inhibition of tumor growth (212). This can either happen directly by binding to angiogenesis inducers in endothelial cells, or indirectly by inhibiting proangiogenic proteins such as epidermal growth factor in tumor cells or tumor-associated stromal cells. Bevacizumab, a monoclonal antibody against tumor-cell derived vascular endothelial growth factor (VEGF) ligand VEGF-A, is one the most successful examples of direct inhibition of angiogenesis, currently widely used in several cancer types (reviewed in (213)).

### **4.2 High throughput drug screening**

High throughput drug screening is a method that allows testing of hundreds of cancer active small molecular compounds for tumor-specific responses (214). Unbiased drug screening provides a useful tool especially for cancers with complex genomic background, as targeting these tumors may require inhibition of multiple molecules (215). Moreover, the screening allows repurposing of drugs that are currently used for other indications. This can be of major significance especially for rare cancers in which clinical trials are challenging to organize.

In addition, the molecular profiling of tumors by RNA or exome sequencing can be incorporated into drug sensitivity data. This affords explanations for drug responses and aids in the search for predictive biomarkers. However, considering the amount of datasets generated in high-throughput screening, efficient bioinformatics tools for analyzing the data are needed.

Previously high throughput drug screening and molecular profiling have been combined successfully in testing clinical blood samples from patients with acute myeloid leukemia (215). Patients were treated with drugs proven effective based on drug sensitivity and resistance testing (DSRT). Interestingly, several clinical responses were seen. Moreover, as the diseases progressed during these therapies and patient samples were re-tested, resistance for the used drugs was detected in the new drug screening. In addition to discovering effective treatments, this method can provide information on mechanisms behind drug resistance. Concerning solid tumors, the challenge lies in constructing representative cell cultures for the drug testing. However, high-throughput drug screening has recently been successfully applied also to patient derived cells from a prostate cancer specimen (216).

## **Aims of the study**

This dissertation project focuses on improving differential diagnostics of AGCTs and exploring treatment options for advanced and recurrent tumors.

The specific aims of the research are to:

- 1) Study the usability of epithelial ovarian carcinoma marker HE4 in differential diagnostics of AGCTs.
- 2) Evaluate the role of TRAIL as a target for therapy via measuring its expression in clinical AGCT patient samples.
- 3) Characterize the hormonal milieu of AGCTs and study the effects of hormonal stimulation in AGCT cells.
- 4) Search for new targeted therapies for AGCT by using high-throughput drug screening combined with molecular profiling of tumors.

## **Materials and methods**

### **1. Patients (I-IV)**

We collected data retrospectively on 138 AGCT patients diagnosed at Helsinki University Hospital from 1956 to 2016. Clinical information was collected from hospital archives and formalin-fixed paraffin embedded samples were used for constructing a tumor tissue microarray. In the prospective cohort starting from 2007, 83 patients signed their informed consent to give blood samples and fresh tumor tissue upon surgery. These patients have been followed using hospital files and all living patients in the prospective cohort have been invited to a follow-up visit in the hospital every 5 and 10 years. The causes of death were collected from death certificates retrieved from the Finnish Causes of Death Registry. The Ethics Committee of Helsinki University Hospital (197/E9/06 and 210/13/03/03/2016)) and the National Supervisory Authority of Welfare and Health in Finland (THL/1469/5.05.00/2012)) approved the study protocol.

### **2. Serum samples (I, II, III)**

Between 2007 and 2016, 153 serum samples from 83 AGCT patients were collected and stored at -80°C for later analyses (Table 1). Thirty three % (n=51) of the samples were collected preoperatively and classified as “with disease” samples, and 67 % (n=102) were collected during the follow-up and classified either “with disease” (WD) or “disease free” (DF). The WD samples were drawn within a month before surgery or chemotherapy, and the DF samples were drawn during follow-up after a minimum of three months from cancer treatment. For controls in study I, 40 preoperative samples from patients with endometrioma and 37 preoperative samples from patients with epithelial ovarian carcinoma were collected from Turku University Hospital. For AGCT and EOC patients, FIGO 2009 classification was used as staging criteria.



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**Table 1.** Clinicopathological data on patients with serum samples. *WD*= with disease, *DF*= disease free, *MP*=menopause

<b>A Patients</b>	<b>Study I (n=82) n (%)</b>	<b>Study II (n=83) n (%)</b>	<b>Study III (n=47) n (%)</b>
Age at diagnosis, years <sup>a</sup>	52 (19-81)	52 (19-81)	56 (26-80)
Tumor stage at diagnosis			
I	76 (93)	77 (93)	47
II	5 (6)	5 (6)	0
III	1 (1)	1 (1)	0
IV	0	0	0
Follow-up time, years <sup>a</sup>	10.7 (2.3-51.3)	8.4 (0.7-49.6)	5.3 (0.4-20.9)
Recurrence			
Yes	21 (26)	21 (25)	19 (40)
No	61 (74)	62 (75)	24 (51)
n/a	0	0	4 (9)

<sup>a</sup> Median (range)

<b>B Sample characteristics</b>	<b>Study I (n=135) n (%)</b>	<b>Study II (n=141) n (%)</b>	<b>Study III (n=51) n (%)</b>
WD	36 (27)	39 (28)	51 (100)
Primary	17 (13)	17 (12)	31 (61)
Recurrent	19 (14)	22 (16)	20 (39)
DF	99 (73)	102 (72)	n/a
Tumor size (WD samples)			
Cm <sup>a</sup>	7.0 (2.0-30.0)	7.0 (2.0-30.0)	7.0 (2.0-32.0)
<10 cm	26 (72)	27 (69)	24 (47)
≥10 cm	9 (25)	11 (28)	15 (29)
n/a	1 (3)	1 (3)	12 (24)
MP status at sample retrieval			
Premenopausal	18 (13)	18 (13)	10 (20)
Postmenopausal	117 (87)	123 (87)	41 (80)

<sup>a</sup> Median (range)

A patient was considered premenopausal if she had one or two ovaries and menopause was not indicated in the medical records (e.g., cessation of regular bleeding, presence of menopausal symptoms, use of hormonal replacement therapy). Patients were considered postmenopausal if both ovaries had been removed (independent of age), or the patient was postmenopausal according to her medical history.

### 3. Tissue samples (II, III, IV)

For the studies II and IV, we used previously constructed tumor tissue microarray (TMA), consisting of 69 primary and 12 recurrent AGCT samples. For the study III, a new TMA was constructed, including 121 primary and 54 recurrent tumor samples, including the samples in the old assay (Table 2). In this new TMA we were able to include both primary and recurrent samples from 19 patients (1-5 recurrent samples each). Both TMAs were paraffin embedded and consisted of quadruple core samples, maximum 95 samples on each slide. Data on both TMAs are summarized in Table 2. All samples were tested positive for *FOXL2* mutation by Taqman SNP Genotyping assay (Applied Biosystems, Warrington, UK). Stage of the primary tumor was determined according to the FIGO 2009 criteria. In part of the TMA sections, some of the core samples were missing from the array, or represented only connective tissue, and could thus not be used in analyses. For control samples, we used three normal ovary samples from premenopausal women who have undergone ovariectomy due to cervical cancer. Fifty-one fresh AGCT tissue samples were collected upon operation between 2007 and 2016. The samples were snap-frozen in liquid nitrogen and stored in -80°C until analyses.

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**Table 2.** Clinicopathological data concerning the tumor tissue microarray (TMA) of AGCTs. The “old” array was used in studies II and IV, whereas a new TMA was constructed for study III.

<b>A Patients</b>	<b>Study II and IV (n=79) n (%)</b>	<b>Study III (n=138) n (%)</b>
Age at diagnosis, years (range)	50 (26-81)	53 (26-81)
Tumor stage at diagnosis		
I	68 (86)	126 (93)
II	8 (10)	8 (6)
III	3 (4)	1 (1)
IV	0	0
n/a	0	3 (2)
Follow-up time, years	15.3 (0.7-38.6)	15.0 (0.7-42.3)
Recurrence		
Yes	27 (34)	51 (37)
No	52 (66)	87 (63)
Survival		
Alive	45 (57)	91 (66)
Dead of AGCT	19 (24)	24 (17)
Dead of other	15 (19)	23 (17)
<b>B Samples</b>	<b>Study II and IV (n=81) n (%)</b>	<b>Study III (n=175) n (%)</b>
Tumor type		
Primary	69 (86)	121 (69)
Recurrent	12 (14)	54 (31)
Histology		
Sarcomatose	31 (38)	72 (41)
Differentiated	50 (62)	98 (56)
n/a	0	5 (3)
Tumor size		
<10 cm	50 (62)	103 (59)
≥10 cm	31 (38)	70 (40)
n/a	0	2 (1)
Nuclear atypia		
High	19 (23)	50 (29)
Low	62 (77)	121 (69)
n/a	0	4 (2)
Mitotic index		
High	21 (26)	48 (29)
Low	60 (74)	123 (71)
n/a	0	4 (2)

## 4. Cell cultures (II-IV)

### 4.1 Primary AGCT cell culture (II-IV)

Between 2007 and 2016, we established primary cultures from 17 primary and 11 recurrent AGCTs. The fresh tumor sample was retrieved straight from operation room in ice cold PBS, mechanically minced, filtered through a 140µm filter mesh with native growth medium DMEM/F12, washed twice with cell culture medium, and incubated for 2-3 days in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 IU/ml). The cells were grown at +37°C in humidified atmosphere containing 5% CO<sub>2</sub>. After incubation cells were trypsinized and plated for experiments. For the hormonal experiments (study III), phenol free medium and charcoal stripped FCS were used. The cells were identified positive for the FOXL2 402C>G mutation by Taqman SNP Genotyping assay (Applied Biosystems, Warrington, UK).

### 4.2 hGL culture (IV)

For the study IV, we used human granulosa luteal cells collected from women undergoing *in vitro*-fertilization treatment in Helsinki University Hospital. Three pooled hGL samples were used, each consisting of granulosa-luteal cells derived from 78–122 mature ovulatory follicles from four to seven different patients. Isolation of hGL cells was performed according to the protocol described by Shi et al (217). The cells were identified wild-type for the FOXL2 402C>G mutation by Taqman allelic discrimination assay. Prior to the DSRT screen, isolated cells were suspended in DMEM/F12 growth medium, supplemented with 2.5% Nu-serum I, ITS+™ Premix (both from BD Biosciences, Bedford, MA, USA), penicillin/streptomycin, and L-glutamine (Gibco).

### 4.3 GCT cell lines (III, IV)

Currently, two commercial cell lines are regarded as human GCT cell lines, KGN and COV434. Of these, KGN is derived from a recurrent adult-type GCT harboring the *FOXL2* mutation, whereas COV434 is wild type for *FOXL2* and represents a juvenile GCT. KGN cell line (a gift from Dr Toshihiko Yanase, Kyushu University, Fukuoka, Japan) was cultured similar to primary AGCT cells. The COV434 cells were cultured in DMEM containing FCS, penicillin and streptomycin.

## 5. Functional experiments (III, IV)

### 5.1 Hormone stimulation and letrozole treatment (III)

To deplete the cells from external hormones, both KGN and primary cultured AGCT cells were grown for 10 or 3 days prior to stimulation, respectively, in phenol red free DMEM/F12 medium supplemented with 10% charcoal stripped FCS, L-glutamine and antibiotics. For the cell viability assay, 10 000 KGN or primary AGCT cells per well were plated on 96-well plates in hormone-depleted medium. For RNA extraction and medium collection, 600 000 cells per well were plated on 6-well plates. 24 h after plating, the cells were treated with 100 nM of FSH (#HOR-253, Immuno Diagnostics, Hämeenlinna, Finland), 1000 nM of E2 (#E2758, Sigma-Aldrich, St Louis, USA), 2 µM testosterone (#86500, Sigma-Aldrich), or 5 µM dose of letrozole (#L6545, Sigma-Aldrich). Cell viability was quantified by the WST-1 assay measuring the amount of metabolically active cells (Sigma-Aldrich), and RNA and medium samples were collected after 96 hours.

### 5.2 Drug sensitivity and resistance testing (IV)

A collection of 230 oncology compounds was used to test responses of primary AGCT cells, human granulosa luteal cells (hGL) and KGN and COV434 cell lines. Compounds were tested in five different concentrations in 10-fold dilutions covering a 10 000-fold concentration range (e.g. 1-10 000 nM). Twenty µl of a single cell suspension (1000 cells/well) was transferred to each predrugged well using a MultiDrop Combi (Thermo Scientific, Waltham, MA, USA) peristaltic dispenser. After 72 h of incubation, the cell viability was measured using the CellTiter-Glo luminescent assay (Promega Corporation, Madison, WI, USA).

KGN and COV434 cell viability was tested in response to the drug combinations in a matrix format. Paclitaxel was used in combination with either dasatinib, everolimus, AZD8055, or PF-04691502. One thousand KGN or COV434 cells were plated per well on predrugged 384-well plates and incubated for 72 h. The cell viability was measured with CellTiter-Glo assay.

## 6. mRNA expression (II, III, IV)

Freshly frozen AGCT tissue samples were lysed in RPI lysis buffer and homogenized using Precellys Lysing Kit and tissue homogenizer (Bertin Technologies, France). The RNA was isolated with Nucleospin RNA/Protein kit (Macherey-Nagel, Düren, Germany) and purified with Nucleospin RNA Clean-up kit (Macherey-Nagel, Düren, Germany) according to instructions. RNA integrity was verified using Agilent 2100 Bioanalyzer (Agilent

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Technologies, Santa Clara, CA, USA). The first-strand cDNA synthesis was performed from 0.5 µg of total RNA by using the Reverse Transcriptase Core Kit (Eurogentec, Seraing, BE).

### 6.1 Quantitative real-time PCR (II, III)

qPCR was carried out using the MESA GREEN qPCR MasterMix Plus for SYBR Assay (Eurogentec, Seraing, BE). Primers listed in Table 3 were designed by using NCBI/Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Target gene expression was normalized to the expression of  $\beta$ -actin (*ACTB*).

**Table 3.** qPCR primer sequences.

Gene	Reference	Oligonucleotide sequence 5'→3'
<i>ACTB</i>	NM_001101.4	F: CTGACGGCCAGGTCATCA
		R: CAGACAGCACTGTGTTGGC
<i>CYP19A1</i>	NM_000103.3	F: GGCATGAGTGATGTGATGGGA
		R: GGCAATTTAGAGTCCTGGTTGA
<i>ESR1</i>	NM_000125.3	F: TGGAGATCTTCGACATGCTG
		R: GCCATCAGGTGGATCAAAGT
<i>ESR2</i>	NM_001040275.1	F: AGAGTCCCTGGTGTGAAGCA
		R: GACAGCGCAGAAGTGAGCATC
<i>FSHR</i>	NM_000145.3	F: TGCCATTCAATGGAACCCAACT
		R: CGTGGAAAAACATCATTAGGCAAT
<i>GPER1</i>	NM_001039966.1	F: ACACACCTGGGTGGACACAA
		R: GGAGCCAGAAGCCACATCTG

### 6.2 RNA-sequencing (III, IV)

Illumina compatible Nextera™ Technology was used for preparation of RNAseq Libraries. Nucleotides were tagged and the fragmented cDNA was purified with SPRI beads. To enrich the library and add the Illumina specific bridge PCR compatible sites, PCR for 5 cycles was performed. PCR products were purified with SPRI beads, and the quality of the library was evaluated by Agilent Bioanalyzer. Cluster generation was performed by C-Bot, and Illumina HiSeq2000 platform was used for paired end sequencing with 93 bp read length. RNA sequencing reads were corrected for adapters and trimmed for quality.

## 6.3 RNA *in situ* hybridization and scoring of the results (III)

RNA *in situ* hybridization was performed on freshly cut 5 µm sections of the TMA using RNAscope 2.5 HD detection kit-BROWN (#322310, ACDBio, Milano, IT) for target mRNA detection. Tissue sections were heated for 1 h at 60°C, then deparaffinized and treated with hydrogen peroxide for 10 min at room temperature. Target retrieval was performed for 15 min at 100°C, followed by protease plus treatment for 15 min at 40°C. The probes Hs-*FSHR* (#400501), Hs-*CYP19A1* (#430861), Hs-*GPER* (#553361), positive control probe Hs-*PPIB* (#313901) and negative control probe *dapB* (#310043) were hybridized for 2 h at 40°C followed by signal amplification steps. The samples were incubated for 60 min with AMP 5 reagent. The sections were next treated with DAB for 10 min at room temperature followed by counterstaining with 50% hematoxylin. The sections were dipped in ammonium water and dehydrated in ethanol series and UltraClear before mounting. Two researchers performed the scoring independently and disagreements were resolved by a joint review. Staining intensity was classified from 0 to 3 (0=negative, 1=weak, 2=moderate, 3=high intensity).

## 7. Protein expression (I-IV)

### 7.1 Immunohistochemistry and scoring of the results (II, III, IV)

Paraffin-embedded tissue sections of 5µm were deparaffinized, rehydrated, treated by boiling for 20 min in 10 mM citric acid for antigen retrieval, and the endogenous peroxidase was blocked with 3 % hydrogen peroxide. Used antibodies are listed in Table 4. Immunoperoxidase staining was performed by using an aviditin-biotin immunoperoxidase system (Vectastain Elite AMB kit, Vector Laboratories, Burlingame, CA, USA) and DAB (Sigma) to visualize the bound antibody. The sections were counterstained by hematoxylin. In studies II and IV, images were produced with LS Leica DMRXA microscope, connected to an Olympus DP70 camera and DCP controller image acquisition program. In study IV images were generated by using 3DHISTECH Pannoramic 250 FLASH II digital slide scanner at Genome Biology Unit (Research Programs Unit, Faculty of Medicine, University of Helsinki, and Biocenter Finland). Two independent researchers performed the scoring by assessing the intensity of staining and percentage of positive cells. The tumors were divided into subgroups of high, intermediate, low and negative staining and the threshold levels were defined individually for each antigen depending on the immunoreactivity and the localization of the antigen. Disagreements were resolved by a joint review. Mitotic index and nuclear atypia of the tumors were estimated by an expert pathologist.

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**Table 4.** Antibodies utilized. IF= immunofluorescence, IHC= immunohistochemistry

Antigen	Manufacturer	Catalog #	Method	Dilution
FOXJ2	Santa Cruz Biotechnology	sc-25825	IF	1:100
TRAIL	Santa Cruz Biotechnology	sc-1891	IHC, IF	1:100 (IHC)/1:50 (IF)
ER $\alpha$	Thermo Clone SP1		IHC	1:25
ER $\beta$	Novocastra	NCL-ER $\beta$	IHC	1:200
CYP19A1	Novusbio	NBP2-33336	IHC	1:5000
survivin	Santa Cruz Biotechnology	sc-10811	IHC	1:400
Akt	Cell Signaling Technology	4691	IHC	1:2000
mTOR	Cell Signaling Technology	2983	IHC	1:50
SRC	Santa Cruz Biotechnology	sc-8056	IHC	1:800
PDGFRA	Santa Cruz Biotechnology	sc-338	IHC	1:1200
PDGFRB	Santa Cruz Biotechnology	sc-339	IHC	1:2000
EPHA5	Santa Cruz Biotechnology	sc-1014	IHC	1:1200

### 7.2 Immunofluorescence (II)

Primary AGCT cells were first cultured for six days and then plated on four-well chamber glasses. After four days, the cells were fixed with 4% PFA, permeabilized with 0.1% PBS-Triton-X, blocked with 1% BSA and stained with primary antibodies. Secondary antibody with Alexa fluor 488 (GFP) conjugate was then added and the nuclei were counterstained with DAPI.

### 7.3 ELISA and automated immunoassays (I, II)

The serum, plasma and cell culture supernatant samples were analyzed using commercial enzyme-linked immunosorbent assay (ELISA) or automated immunoassays according to manufacturer's instructions. The following kits for ELISA were used: HE4 (Fujirebio Diagnostics inc., Malvern, PA, USA), inhibin B (Gen II ELISA, Beckman Coulter), AMH (AnshLabs, Webster, TX, USA), TRAIL (R&D Systems, Minneapolis, MN, USA). Automated immunoassays of the hospital clinical laboratory were used for FSH (Abbott Laboratories, IL, USA) and E2 (Siemens Healthcare, Erlangen, Germany).

### 7.4 Liquid chromatography tandem mass spectrometry (LC-MS/MS) (III)

LC-MS/MS was utilized for assessing estradiol concentrations in cell culture supernatants after hormone stimulations. Medium was collected from KGN and primary AGCT cells at 96 h time point and analysed for E2 concentration using a mass spectrophotometer at



HUSLAB. Calibrators containing 25-1000 pmol/l of E2 (Cerilliant) were prepared in 50% methanol. Forty µl of sample extracts and calibrators were analysed on an LC-MS/MS system equipped with an AB Sciex 5500 triple quadrupole mass spectrometer. Data were acquired and processed with the Analyst Software (Ver 1.6.2; AB Sciex).

### 8. Statistical analyses (I-IV)

The cell culture and mRNA expression data were analysed with one-way ANOVA followed by with-control Dunnett's or each pair Student's t-test. Serial measurements in serum expression data with parametric distribution according to Shapiro-Wilk's test were analyzed with generalized linear model and Pearson's correlation coefficient. Parameters with nonparametric distributions even after logarithmic transformation were analyzed with Mann-Whitney test or Spearman's rho. For multiple regression, generalized linear model and maximum likelihood ratio estimation was used. For serum marker comparisons, receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) values were calculated. The immunohistochemical data and categorical variables were analysed with contingency tabling (2x2) and chi-square or Fisher's exact tests when appropriate. The comparison between primary and recurrent tumors was performed by matched-pair t-test analysis. Survival curves of different groups were illustrated by Kaplan-Meier plots and compared with the Log-rank test.

To quantify the drug responses of individual samples and to compare the drug responses between the AGCT samples, the model-based drug sensitivity score (DSS) was calculated for the drug sensitivity and resistance tested samples (218). Clustering of the drug sensitivity profiles across the AGCT and control samples was achieved using Ward's unsupervised hierarchical clustering method with Euclidean and Manhattan distance measures for the drug and sample profiles, respectively, utilizing heatmap.2 function in "gplots" R package. The associations between the DSS profiles were analyzed with Spearman's rank correlation coefficient. Drug combination synergy was calculated by comparing the observed joint inhibition level at each dose combination to the expected combination effect using the zero interaction potency model (219).

The RNA sequencing data in study III were processed and analyzed with SePIA (220) on the Anduril framework (221). Reads were quantified with Kallisto, and edgeR was used for differential expression analysis. In study IV the sequencing data were mapped to human reference genome Build 37 using Tophat2 and assigned to genomic features (Ensembl 69 gene models) using Cufflinks and HTseq with default settings. Variant calling and filtering from RNA sequencing reads was performed on the AGCT samples using Genome Analysis Toolkit (GATK).

Two-sided p-value less than 0.05 was considered statistically significant. Data in studies I-III were analyzed using JMP pro13 and in study IV using the R 3.1.0 statistical software platform.

## Results and discussion

### 1. Searching optimal diagnostic tumor marker for AGCT (I)

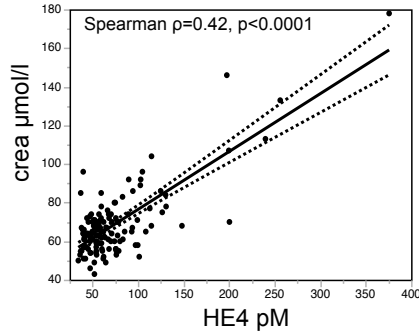
The exact diagnosis of ovarian mass cannot be made until histopathological evaluation of the tumor. Preoperatively, however, it is important to distinguish potentially malignant tumors from benign ovarian cysts. To achieve optimal treatment results, ovarian cancer should be operated in specialized units by experienced surgeons. This applies also to AGCT, as successfully performed surgery is the cornerstone of treatment and intraoperative tumor rupture increases the risk for recurrence (8, 81).

In the diagnostics of most common ovarian malignancies, epithelial carcinomas, CA125 and HE4 appear as most precise tumor markers and they are widely used in preoperative evaluation of ovarian tumors. Concerning AGCTs, inhibin B and AMH have emerged as sensitive and specific markers in surveillance (29). There are some reports regarding CA125 in AGCTs, however no data exist on other epithelial serum tumor marker, HE4, in this tumor type. We thus aimed to measure serum levels of HE4 in AGCT patients. We also aimed to clarify which tumor marker or marker combination is most sensitive and specific in differentiating AGCT from other ovarian tumors.

#### 1.1 Serum HE4 levels are not elevated in AGCTs

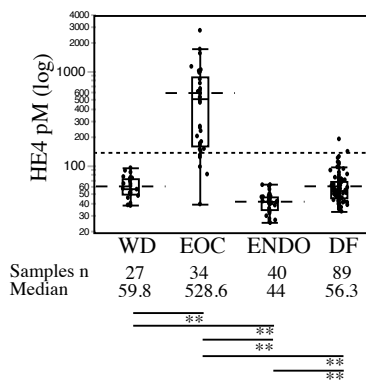
HE4 levels were measured in 36 preoperative AGCT samples (“with disease” samples, WD) (Table 1 in Mat & Met). The median level was 73.3 pM (range 39.6-256.1 pM). When the cut-off for normal values was set to 150 pM according to the kit manufacturer’s instructions, only one AGCT WD patient had serum concentration above this level. Regarding this patient, the elevated HE4 value was putatively due to impaired renal function. It is widely known, that HE4 concentrations are increased in chronic kidney disease (24, 222). This was also evident in our data, as HE4 correlated positively with the creatinine levels in AGCT patients (Figure 6). HE4 levels in AGCT WD group did not correlate to tumor size, stage or risk of recurrence.

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**Figure 6.** Serum HE4 levels correlated positively to serum creatinine (crea) levels in AGCT patients. HE4 levels were measured in 36 AGCT WD patients and 99 AGCT DF patients.

Next, we compared the serum HE4 levels of AGCT WD patients with AGCT patients with no macroscopic disease (“disease free” samples, DF). There were altogether 99 samples from 76 patients, as the sample set included two serial follow-up samples from 25 patients. Surprisingly, there was a significant difference between these two patient groups, the AGCT WD group presenting with higher HE4 values ( $p=0.034$ ). Nevertheless, after excluding patients with impaired renal function, no difference was detected ( $p=0.35$ , Figure 7). This confirms our finding on normal HE4 levels in AGCT.



**Figure 7.** Serum HE4 levels in AGCT were not elevated. HE4 levels were significantly increased in EOC compared to AGCT WD, AGCT DF and ENDO groups. In the ENDO group the levels were significantly lower than in the other groups, due to younger age distribution in the ENDO group. Patients with impaired kidney function ( $GFR < 60 \text{ ml/min/1.73m}^2$ ) were excluded. The dashed line indicates the cut-off value (150 pM).  $***=p < 0.0001$ . WD=with disease, EOC=epithelial ovarian carcinoma, ENDO=endometrioma, DF=disease free.

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### 1.2 Low HE4 levels differentiate AGCTs from EOCs

HE4 levels in AGCT patients were also compared with preoperative levels in 37 epithelial ovarian carcinoma patients (EOC). As expected, the levels were significantly higher in the EOC group ( $p < 0.0001$ ). This was also seen when patients with renal dysfunction were excluded (Figure 7). In our study most EOCs (81%) were stage III and IV whereas all AGCTs were stage I. This reflects the common tumor stage at diagnosis, due to the different growth type of these two distinct tumor types. Some earlier studies have presented HE4 as a sensitive marker also in stage I EOC (23, 223), nevertheless later studies have not confirmed this finding (224). The difference between AGCT and EOC patients remained significant in this study also when only stage I patients were analyzed (data not shown).

When the HE4 levels were compared between AGCT WD samples and 40 preoperative samples from endometrioma patients (ENDO), the difference was statistically significant ( $p < 0.0001$ ). This difference was also noted when patients with impaired renal function were excluded (Figure 7). Apart from renal function, age is considered as the main factor in determining HE4 levels in healthy subjects (25). AGCT and ENDO groups differed significantly on patient age: the median age in AGCT group was 60 years (range 36-80 years) whereas in ENDO group it was 32 years (range 26-47 years). According to a study determining HE4 limits in Nordic population, HE4 serum levels were 35% higher in the age of 60 when compared to the age of 30 and our results are convergent with this data. In the study by Bolstad et al (25), age dependent reference limits are suggested and this is also supported by another report from Korea (225). This has lead to use of separate reference values that take into account patient's age. For example in our clinic the limit for patients  $< 50$  years is 70 pM and for patients  $> 50$  years it is 90 pM. If these reference levels would have been used in our study, a majority (72%) of AGCT WD samples would be classified as normal. Of the patients whose HE4 values exceeded the clinical reference limit, 90% either had impaired renal function or were smokers. Smoking has been shown to increase HE4 levels even up to 29% (25). HE4 is expressed in the airway epithelium and it is suggested that airway inflammation could explain increased levels (25, 226). In our cohort, we lacked the information of smoking status from most patients, and thus could not properly estimate the effect of smoking in this data set.

In addition to the respiratory tract, HE4 is normally expressed in breast, kidney and epithelium of reproductive tissues, including endocervical and endometrial glands and fallopian tubes (227, 228). Earlier, it has been reported that HE4 is not detected in AGCT tissue at protein level, however this was based on staining of only five AGCTs (226). Nevertheless, our results on HE4 serum concentrations support this finding.

### 1.3 CA125 is elevated in a subset of AGCTs without any prognostic significance

Serum CA125 levels were analyzed in preoperative samples of AGCT patients, and the median value was 19.5 IU/ml (range 5-5400 IU/ml), which is clearly below the common

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reference limit. Contrary to HE4, the reference limits in CA125 are more established and 35 IU/ml is widely used as the upper limit. Even though the levels were generally low, they were significantly higher in AGCT WD patients when compared with AGCT DF patients. Concerning one AGCT patient with elevated CA125 levels, there was also simultaneous endometriosis which may have influenced the CA125 value. Another AGCT patient had extremely high CA125 concentrations: 5400 IU/ml in WD sample and 696 IU/ml in DF sample. No explanations for this were evident. The AGCT WD levels were similar to levels in ENDO patients and as expected, in EOC patients CA125 was significantly elevated compared with other groups. Altogether, only 25% of AGCT WD patients had CA125 levels above normal limit, which is in line with results from earlier studies (27, 28). No correlation between serum CA125 level and tumor size or tumor stage were noted and the levels were not prognostic for disease-specific or overall survival.

### 1.4 Inhibin B and AMH are specific markers for AGCT

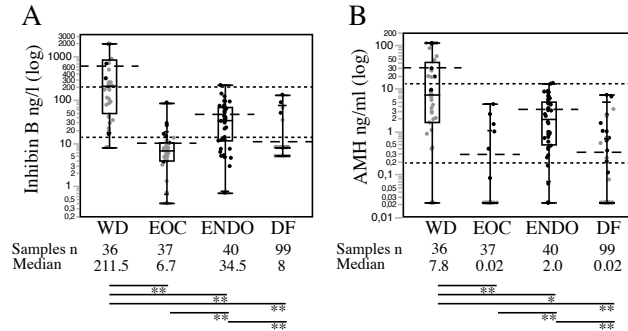
Inhibin B and AMH are both produced by AGCT and are validated markers in AGCT surveillance. We now assessed their role in differential diagnostics.

The median concentration in AGCT WD group for inhibin B was 211.5 ng/l (range 8-2000 ng/l), and the levels were significantly higher when compared to EOC and ENDO groups ( $p < 0.0001$ ) (Figure 8A). In the ENDO group, inhibin B was elevated when compared with the EOC and AGCT DF groups. This is due to fact that all the patients in the ENDO group were premenopausal and had functional ovaries. Inhibin B is normally secreted from granulosa cells of developing follicles, and thus its levels at fertile age are physiologically higher than in the postmenopause where the concentrations become undetectable. For premenopausal women the reference limit in our study was 200 ng/l, whereas in postmenopausal women levels  $>16$  ng/l were considered abnormal. From the 36 AGCT WD patients 33 (92%) had inhibin B levels above the reference limits. Inhibin B has earlier been studied in endometriosis patients, and the levels have been reported to be lower than in healthy individuals possibly due to impaired granulosa cell function (229). In our ENDO group only one patient presented with an abnormal inhibin B value (220.7 ng/l). In the EOC group, none of the patients had levels above the reference limit. This is in line with earlier findings on normal inhibin B levels in serous tumors (230). Inhibin B has however been reported to increase in mucinous tumors (230). The EOC group in our study included two mucinous carcinomas exhibiting normal inhibin B levels. It is notable, that some other sex-cord stromal tumors may present with increased inhibin B levels, including benign fibromas and thecomas (230, 231).

AMH levels were also significantly higher in AGCT WD patients when compared to those of EOC and ENDO patients. Median AMH concentration in AGCT WD was 7.8 ng/ml (range 0.02-125 ng/ml) (Figure 8B). Similar to inhibin B, also AMH is produced by normal granulosa cells of growing follicles, especially from primary up to small antral stage. The levels peak around the age of 25 and decline then gradually during fertile age span,

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becoming undetectable in menopause. The upper reference limit for AMH was 13  $\mu\text{g/l}$  and  $<0.2 \mu\text{g/l}$  for premenopausal and postmenopausal patients, respectively. In the AGCT WD group, 83% of the patients had elevated AMH levels, in line with earlier studies reporting AMH to be elevated in 78-92% of AGCTs (29, 31, 32, 77). In the EOC group, none of the patients had elevated AMH levels, and in the ENDO group only two patients had AMH levels exceeding the reference limit.

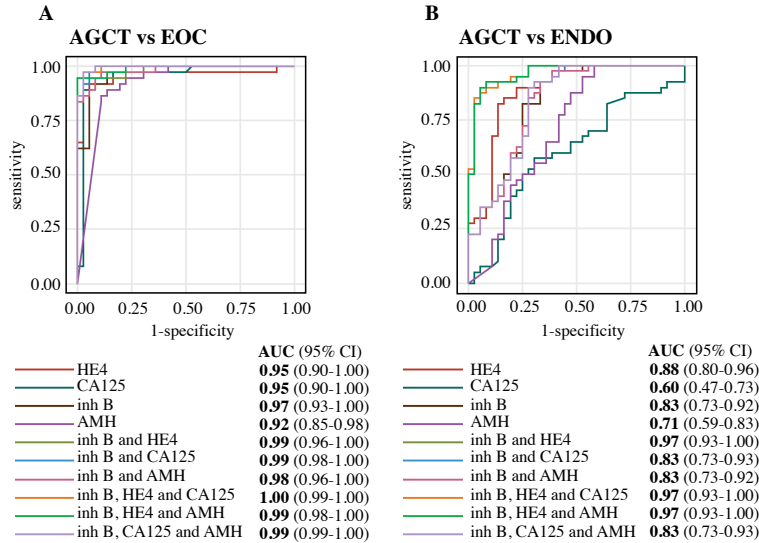


**Figure 8.** Serum concentrations for inhibin B (A) and AMH (B). The black and gray dots represent premenopausal and postmenopausal patients, respectively. Dashed lines indicate the cut-off levels. \*= $p < 0.05$ , \*\*= $p < 0.0001$ . WD=with disease, EOC=epithelial ovarian carcinoma, ENDO=endometrioma, DF= disease-free.

### 1.5 Inhibin B is the most accurate single preoperative marker in AGCT differential diagnostics

We also performed receiver operating characteristic (ROC) curve analyses by using the continuous values of the markers (Figure 9). In distinguishing AGCTs from EOCs, all single markers were highly accurate, resulting in AUCs between 0.92 and 0.97. Inhibin B performed as the most precise single marker with AUC 0.97. In differentiating AGCTs from ENDOs, the accuracies of single markers were lower (0.60-0.88), reflecting the irrelevancy of HE4 and CA125 in both AGCTs and endometriomas, and the normal variation of inhibin B and AMH in premenopausal women.

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**Figure 9.** The receiver operating curve analyses for HE4, CA125, inhibin B, AMH, and marker combinations using continuous values. The accuracies of single markers and combinations of markers in distinguishing between AGCT WD and EOC (A) and AGCT WD and ENDO (B). WD=with disease, EOC= epithelial ovarian carcinoma, ENDO=endometrioma, DF=disease free.

ROC curve analyses were also performed by using the dichotomized data regarding the cutoff levels for each marker. In differentiating AGCTs from EOCs, all the markers performed well, inhibin B presenting as the most accurate single marker with AUC 0.96. In differentiating AGCTs from ENDOS, the utilization of the cut-off levels improved the accuracy of inhibin B and AMH (AUC 0.95 and 0.89, respectively). The ROC-AUC comparison analyses using the dichotomized data confirmed the superiority of inhibin B in AGCT differential diagnostics, as there was no added value to use marker combinations compared to inhibin B measurements alone, when differentiating between AGCTs and EOCs. In differentiating AGCTs from ENDOS, inhibin B and AMH were equally accurate. Combination of inhibin B and AMH was significantly more accurate than inhibin B alone ( $p=0.036$ ) or AMH alone ( $p=0.004$ ). The combination resulted in sensitivity of 100% and specificity of 93%.

This was the first study investigating circulating HE4 levels in AGCTs. The increased HE4 levels in patients with impaired renal function underline the importance of measuring creatinine levels if elevated HE4 levels are detected. CA125 is elevated in only subset of AGCTs without any clinical correlations. Clinically, it is important to note that normal CA125 and HE4 levels do not rule out a malignant ovarian tumor. Inhibin B is a sensitive and specific marker in AGCT differential diagnosis and combination of AMH and inhibin B may be useful especially in premenopausal patients.

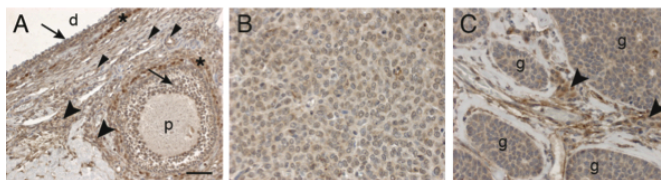
## 2. TRAIL as a potential therapeutic agent for AGCT (II)

Disturbances in apoptosis are suggested to play a crucial role in AGCT pathogenesis. Our group and others have earlier confirmed the activity of TRAIL pathway in AGCT and demonstrated the ability of TRAIL to induce apoptosis in AGCT cells (193, 232). Now we aimed to translate this to clinical patient samples and evaluated the levels of TRAIL in serum and tumor specimen of AGCT patients. Physiologically TRAIL is expressed in various cell types, particularly in the immune cells, and soluble TRAIL has been detected in circulation under physiological conditions (144, 233-235). Recent data from animal studies imply that lack of TRAIL may promote pathological processes such as chronic inflammation and tumorigenesis (236).

### 2.1 TRAIL protein expression is decreased in large AGCTs

First, we immunostained our normal ovary samples with TRAIL antibody and discovered positive expression in granulosa and theca cells of primary and preovulatory follicles. Endothelial cells and ovarian stromal cells also showed positive staining for TRAIL (Figure 10A).

Immunohistochemical analysis of 83 AGCTs revealed TRAIL protein expression in 48 (59%) of studied tumors at a comparable level to normal granulosa cells. Staining pattern was diffuse and tumor stroma presented even stronger staining compared to tumor cells (Figure 10 B,C). Thirty (37%) of AGCT tumors showed low staining and three (4%) tumors remained negative for TRAIL. Interestingly, TRAIL expression was significantly lower in tumors over 10cm in diameter when compared to smaller tumors ( $p=0.04$ ). We also noted a positive correlation between expressions for TRAIL and for TRAIL receptors DR4 ( $p=0.006$ ) and DR5 ( $p=0.02$ ), confirming the functional TRAIL pathway in AGCTs. Noteworthy, TRAIL expression correlated positively to GATA4 expression in AGCTs ( $p=0.003$ ). This is of interest as GATA4 has earlier been reported to protect AGCT cells from TRAIL-induced apoptosis in both KGN cell line and primary AGCT cultures (193). Thus, our results suggest resistance of TRAIL mediated apoptosis by increased GATA4, leading to enhanced tumor growth.



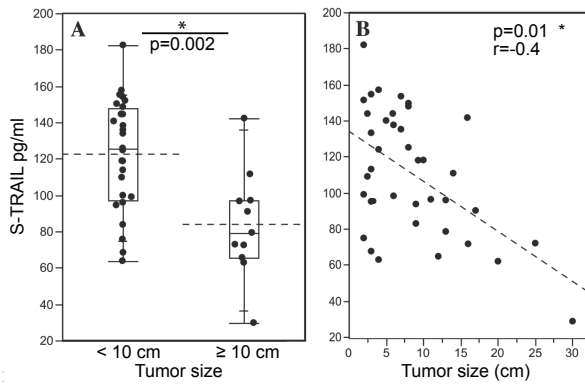
**Figure 10.** Positive IHC-staining for TRAIL in the normal ovary (A) and in AGCT (B, C). TRAIL protein was expressed in granulosa cells (arrows), thecal cells (asterisks), in endothelial cells (small arrowheads) and in the ovarian stroma (big arrowheads) (A). Diffuse staining in AGCT (B). Staining was stronger in tumor stroma than in the tumor cells (C). d=dominant follicle, p=small secondary follicle, g=GCT cells



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### 2.2 Circulating TRAIL levels are lower in patients with large AGCTs

To clarify the role of circulating TRAIL levels in AGCT patients, we next analyzed serum TRAIL concentrations from 141 serum samples (Table 1). Of these, 39 samples were drawn preoperatively (with disease, WD samples) and 102 samples were drawn at least a month after radical surgery with no evident of disease (disease free, DF samples). We noticed that the tumor itself had minimal effect on circulating TRAIL levels, as no significant difference between WD and DF patient groups emerged. Nevertheless, soluble TRAIL levels were significantly lower in patients with tumors more than 10 cm in diameter (mean 83.6, SD 29.2, pg/ml) compared to patients with tumors less than 10 cm in diameter (mean 122.1, SD 30.2, pg/ml,  $p=0.002$ ) (Figure 11A). This was confirmed in analysis regarding tumor size as a continuous variable ( $r=-0.4$ ,  $p=0.01$ ) (Figure 11B). Tumor size did not correlate to any patient derived factors as age, body weight or blood leukocyte counts.



**Figure 11.** Circulating TRAIL levels in AGCT patients were decreased in larger tumors. Serum TRAIL levels significantly differed between tumors < 10 cm in diameter and tumors ≥ 10 cm in diameter (A). Serum TRAIL levels correlated negatively to tumor diameter (B).

### 2.3 TRAIL levels in AGCTs are related to estradiol levels

Circulating TRAIL levels were recently investigated in a healthy population, and interestingly in women under 50 years of age serum TRAIL levels were significantly lower than in older women and in age-matched men (233). This discovery led to monitoring of both TRAIL and estradiol levels in women, demonstrating inverse correlation between TRAIL and estradiol levels in the sera. TRAIL levels were the lowest in pregnant women, whereas in prepubertal girls and in postmenopausal women TRAIL concentrations were

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significantly higher. This was also supported by *in vitro* studies demonstrating downregulation of TRAIL production by estradiol (233).

As AGCTs are known to produce estradiol, we investigated the potential effect of serum estradiol on TRAIL levels. In our AGCT cohort, circulating TRAIL concentrations associated negatively with serum estradiol levels (Spearman's Rho -0.32,  $p=0.05$ ), whereas no correlations were found between TRAIL and patient's menopausal status, FSH or LH levels. Regarding estradiol levels, positive correlation to tumor size was also noted ( $r=0.41$ ,  $p=0.01$ ). Furthermore, tumor size was associated to FSH ( $p<0.0001$ ) and LH ( $p<0.0001$ ) levels and menopausal status ( $p=0.01$ ).

### 2.4 Tumor size correlates negatively to circulating TRAIL concentrations

According to univariate analyses, tumor size and estradiol levels affected TRAIL levels whereas tumor size was further associated with menopausal status and hormonal factors. To clarify the significance of independent factors influencing circulating TRAIL levels, a multivariate analysis was conducted. According to this analysis, tumor size was the only factor independently affecting circulating TRAIL levels ( $p<0.001$ ). Altogether, increased estradiol levels are a consequence of increased tumor size and do not directly regulate TRAIL in AGCT patients. Our results suggest that tumor stroma and infiltrating blood and immune cells may contribute to TRAIL levels in the circulation. In larger tumors the significance of stromal TRAIL production is decreased, leading to decreased apoptosis in tumor cells expressing TRAIL receptors. Regulatory mechanisms thus seem to decline in the course of AGCT growth.

### 2.5 Therapeutic potential of TRAIL

TRAIL is able to induce apoptosis mainly in malignant cells, and therefore it has emerged as a promising therapy for various tumor types. Both soluble TRAIL and antibodies against TRAIL receptors, especially TRAIL-R2 specific agonistic antibodies, have shown antiproliferative effects both *in vitro* and *in vivo* (reviewed in (237)). Moreover, TRAIL has been shown to act as an effector molecule in immunotherapy (238). In clinical trials, TRAIL agonists have been tested as a monotherapy in hematological malignancies, non-small cell lung cancer and colorectal cancer (239-241). However, the efficacy of this therapy has been disappointing: even though TRAIL-R agonists have been well tolerated, their anticancer properties in patients have been very modest, and many TRAIL targeted therapies have been dropped out from clinical development. There are a few identified problems in TRAIL based targeting. Even though most cell lines have been sensitive to TRAIL, many primary tumor cells have appeared either partially or completely resistant to TRAIL monotherapy. As the potential hepatotoxicity of TRAIL was worrisome, the first regimens tested have had only suboptimal agonistic potency, thus possibly explaining the limited clinical efficacy. Another important issue has been the lack of predictive biomarkers in TRAIL treated patients. There

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have also been shortcomings in stability and bioavailability of TRAIL targeting compounds (reviewed in (237)). Moreover, it has been demonstrated that TRAIL itself may also have pro-tumorigenic effects such as activation of certain tumor promoting signalling pathways (242, 243).

Recently, there have been significant advances also in development of TRAIL targeted therapies. Stability and pharmacokinetic properties of recombinant TRAIL have been improved (244). A couple of recent studies found that the combination therapy of soluble TRAIL and TRAIL-R2-specific antibody had high efficacy and with this treatment some previously resistant cell lines were sensitized to TRAIL (245, 246). TRAIL has also been demonstrated to have a role in reversing multidrug resistance (247). Furthermore, co-targeting tumor immune system has been proposed to enhance efficacy of TRAIL targeting treatments (234, 248, 249).

Concerning AGCTs, a phase I study assessing TRAIL-R2 receptor agonistic antibody reported clinical response including 23% reduction in tumor size in a patient with advanced AGCT (239). Even though the response is modest, it can be regarded as promising, while more potent TRAIL targeting compounds are being developed. Recent studies report increased TRAIL efficacy when combined with cisplatin or other traditional therapeutics (250, 251). Also in AGCT cells the efficacy of TRAIL has been shown to be increased by cisplatin (232). We thus suggest that TRAIL targeted therapies should be clinically tested in combination with traditional chemotherapeutics or other targeted treatments.

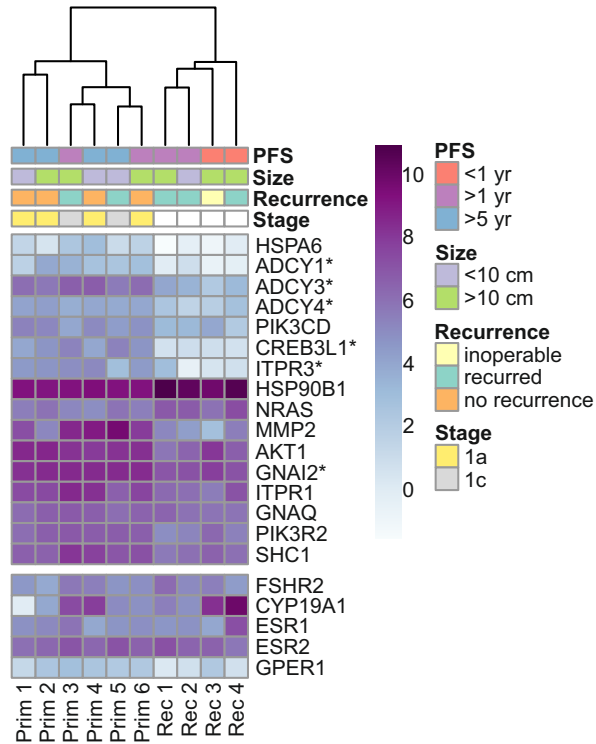
### 3 Characterization of FSH and E2 signaling in AGCTs (III)

AGCTs are described as hormonally active tumors, due to their ability to secrete hormones such as estradiol and inhibins (32, 36). These tumors have also been reported to express hormonal receptors, however this has only been studied in small patient cohorts (175, 178, 252-254). To date, hormone therapies have been used in the treatment of relapsed AGCTs, even though the scientific basis for this is lacking. It is of major clinical significance whether hormones, especially estrogens, promote tumorigenesis of AGCTs. We used our large *FOXL2* mutation validated data set to study the expression levels of hormone receptors and aromatase enzyme and to investigate whether these factors have any prognostic role in AGCTs. We also examined the effects of hormonal stimulations on primary cultured AGCT cells and KGN cell line.

#### 3.1 Primary and recurrent AGCTs show distinct transcriptional profiles

First, we performed RNA sequencing of ten AGCTs (six primary and four recurrent tumors). Altogether 1091 genes were differentially expressed between primary and recurrent tumors. Only six of these genes were involved in estrogen signaling: three adenylate cyclase genes contributing to formation of cAMP in response to G-protein signaling, and CREB3L1, encoding for cAMP responsive element, showing higher expression in primary tumors compared with recurrent tumors (Figure 12). However, when analyzing the hormonal pathway genes (KEGG estrogen signaling hsa04915) using unsupervised hierarchical clustering, the primary and recurrent tumors clustered separately, indicating distinct transcriptional profiles.

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**Figure 12.** Transcriptional profiling of six primary and four recurrent AGCTs. Heatmap showing the expression levels of differentially expressed estrogen signaling genes between primary and recurrent samples (upper part). Genes with statistically significant differential expression ( $p < 0.05$ ,  $FDR < 0.25$ ) are marked with an asterisk. Lower part of the heatmap presents scaled expression levels of key hormonal signaling genes in primary and recurrent AGCTs.

### 3.2 FSHR is widely expressed in AGCTs, whereas CYP19A1 is detectable only in subset of AGCTs

Expression levels of hormone receptors and aromatase enzyme (CYP19A1) were studied first by RNA seq in above mentioned ten tumors and these findings were further validated by using our newly constructed tumor tissue microarray. TMA consisted of 175 AGCTs, all verified positive for *FOXL2* mutation (Table 2 in Mat & Met section).

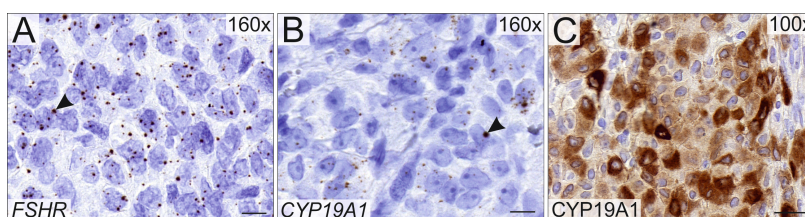
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According to the RNA seq data, *FSHR* was robustly expressed in all AGCTs showing only little variation between individual tumors (Fig 12). In the TMA, *FSHR* mRNA was detected in majority (90%) of AGCTs and the signal was strong or moderate in 60% of the tumors (Figure 13A and Table 5). *FSHR* expression was lower in tumors with high mitotic activity ( $p=0.01$ ), but the expression did not correlate with other clinical parameters such as tumor size, stage, or risk of recurrence. Our results thus confirm the robust expression of *FSHR* in AGCTs.

FSH has long been proposed to have a role in AGCT pathogenesis (169, 255). The gene expression profile of AGCT has been demonstrated to be similar to FSH-stimulated granulosa cells and *FSHR* has earlier been detected in minor AGCT cohorts (169). Now, for the first time the expression was confirmed in a large cohort. *FSHR* belongs to the group of G-proteins, which have shown to harbor oncogenic mutations in other endocrine tumors (256). Fuller et al investigated 15 AGCTs for potential activating mutations on *FSHR* genes but could not detect any mutations or polymorphisms (257). There is strong evidence existing that *FSHR* is functional in this tumor type, as genes responding to FSH stimulation (including protein kinase A and cyclin D2) have been shown to be highly expressed in AGCT (169). On the contrary, expression of LHR, another gonadotropin receptor, is low in AGCTs.

As AGCTs are known for their ability to produce estrogen, we hypothesized aromatase expression to be high in AGCTs. Surprisingly, *CYP19A1* expression varied markedly among the tumors, the scale varying between -0.07-9.85 Log2tpm (Fig 12). In the TMA, CYP19A1 immunoreactivity was detected in less than half (48%) of the tumors, and only 17% showed moderate or strong staining (Figure 13C, Table 5). Next, the expression of *CYP19A1* was assessed at mRNA level in the TMA (Figure 13B, Table 5). The RNA *in situ* hybridization revealed that only 15% of the tumor specimens are positive for *CYP19A1*. Thus, the expression levels were significantly lower than in earlier reports (171, 172). Especially the number of AGCTs expressing *CYP19A1* mRNA was unexpectedly low and may partly be explained by the short half-life of *CYP19A1* mRNA (258). Nevertheless, *CYP19A1* mRNA expression correlated significantly with the moderate/high CYP19A1 protein expression ( $p<0.0001$ ). CYP19A1 may require constitutive FSH stimulation, which is suppressed in AGCT patients through negative feedback of the pituitary by tumor-derived inhibin B. Additionally, another factor secreted by AGCTs, AMH, has been shown to attenuate FSH-mediated stimulation of CYP19A1 expression (259). Thus, AGCTs seem to exhibit self-limiting regulation of CYP19A1 expression and estradiol synthesis.

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**Figure 13.** *FSHR* and *CYP19A1* expression in AGCT TMA, assessed by RNA in situ hybridization (A,B). *CYP19A1* protein expression was assessed by immunohistochemistry. Representative images of high (1,3) and intermediate (2) staining patterns in AGCTs. Magnifications 160 x (A,B) and 100 x (C) and scale bars 20µm (A,B) and 10 µm (C). Arrows indicate positively stained cells.

**Table 5.** Marker distributions for *FSHR* and *CYP19A1* in TMA. <sup>a</sup> = RNA in situ hybridization and <sup>b</sup> = IHC.

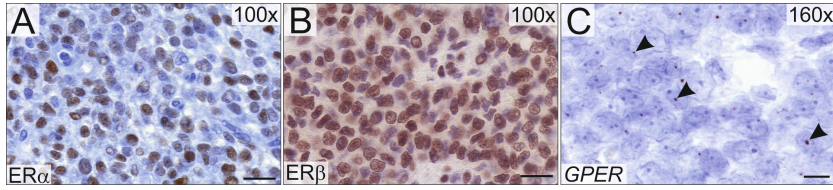
Marker	Negative n (%)	Weak n (%)	Intermed n (%)	High n (%)
<i>FSHR</i> n=165 <sup>a</sup>	17 (10)	49 (30)	81 (49)	18 (11)
<i>CYP19A1</i> n=167 <sup>a</sup>	142 (85)	13 (8)	12 (7)	0
<i>CYP19A1</i> n=156 <sup>b</sup>	81 (52)	49 (31)	17 (11)	9 (6)

### 3.3 ER $\beta$ expression is stronger in recurrent tumors

We then elucidated the expressions of two nuclear estrogen receptors ER $\alpha$  and ER $\beta$ , and of membranous receptor *GPER1*. Of the estrogen receptors, *ESR2* was the most highly expressed (range 5.75-7.15 Log2tpm), whereas *GPER1* expression levels were generally low at the mRNA level (range 0.20-2.84 Log2tpm).

The protein staining patterns of ER $\alpha$  and ER $\beta$  were studied in the TMA. Even though ER $\alpha$  and ER $\beta$  are active in the nucleus, they can also be detected in the cytoplasmic compartments of cells. ER $\alpha$  immunoreactivity was observed in 32 % of the tumors, which is in line with previous results (175) (Figure 14A, Table 6). In the ER $\alpha$ -positive tumors, the staining pattern was nuclear in 59 % and cytoplasmic in 48 % of the tumors. ER $\beta$  protein was detected in 93% (Figure 14B, Table 6) and localized merely to the nuclei. The staining intensity was classified as moderate or strong in 67 % of the tumors. *GPER1* mRNA expression was detected only in 14 % of the TMA tumors by RNA *in situ* hybridization (Figure 14C, Table 6).

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**Figure 14.** Expressions for estrogen receptors in TMA. High immunohistochemical staining pattern of ER $\alpha$  and ER $\beta$  (A,B) and weak RNA in situ staining pattern of GPER (C). Magnifications 100 x (A,B) and 160 x (C) and scale bars 10 $\mu$ m (A,B) and 20  $\mu$ m (C). Arrows indicate positively stained cells.

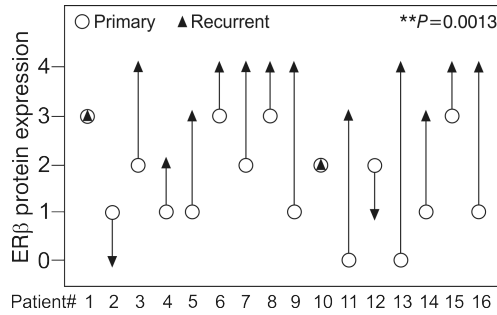
**Table 6.** Marker distributions for estrogen receptors in TMA. a = IHC, b= in situ hybridization. Regarding ER $\alpha$  immunostaining, both nuclear and cytoplasmic staining were detected.

Marker	Negative n (%)	Weak n (%)	Intermed n (%)	High n (%)
ER $\alpha$ (n=165) <sup>a</sup>	113 (68)	19 (12)	33 (20)	0
ER $\beta$ (n=152) <sup>a</sup>	9 (6)	40 (26)	40 (26)	63 (41)
GPER1 (n=161) <sup>b</sup>	139 (86)	22 (14)	0	0

Interestingly, ER $\beta$  expression levels were significantly higher in recurrent AGCTs when compared to primary tumors ( $p=0.001$ ). Furthermore, paired analysis of primary and recurrent samples from same patients demonstrated a significantly stronger ER $\beta$  immunoreactivity in the recurrent tumor ( $p = 0.0013$ ) compared to patient's primary tumor (Figure 15). A similar pattern between primary and recurrent samples was noted for GPER1 mRNA expression, although the levels of expression were low. The expression levels of ER $\alpha$  in recurrent samples did not differ from that of primary samples. Interestingly, CYP19A1 expression in AGCTs correlated positively to ER $\alpha$  protein expression ( $p=0.009$ ). This may suggest a functional role for ER $\alpha$  in a subset of AGCTs. Nevertheless, none of the estrogen receptors had prognostic significance in terms of disease-free or overall survival, nor was there any correlation between estrogen receptor status and tumor size, stage at primary diagnosis, recurrence rate, or menopause status of the patient.



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**Figure 15.** *ERβ protein levels are increased in recurrent AGCTs compared with primary tumors. Paired analysis of the expression of estrogen receptor ERβ in 16 patients with both primary and recurrent tumor samples in the TMA. On the y-axis intensity of ERβ is presented, 0=negative and 4=strongest intensity.*

Our results are in line with earlier reports confirming that ERβ is the predominant estrogen receptor in AGCT (175, 178, 254). The stronger intensity in recurrent tumors suggests a role for ERβ in AGCT pathogenesis. The expression level for ERα has been consistent in several studies. However, levels of GPER1 expression were markedly lower than in earlier reports, questioning the specificity of antibodies used in earlier studies (113, 181).

### 3.4 FSH levels are low in AGCT patient sera

In addition to determination of tissue expression of hormone receptors, we assessed the hormone levels in patient sera. The peak incidence of AGCTs is during menopause, at a time when FSH levels physiologically increase. However, FSH levels in AGCT patients are usually low, even in the postmenopausal patients. This is thought to be caused by regulation of pituitary secreted FSH by tumor-derived hormones such as E2 and inhibin B.

Here, we analyzed FSH, E2, and inhibin B levels in 51 preoperative serum samples. FSH levels were averagely low in AGCT patients, with median levels of 1.55 IU/l (range 0.05-15.3 IU/l) and 6.6 IU/l (range 0.1-60.3 IU/l) in premenopausal and postmenopausal patients, respectively. For E2 the median level was 0.125 nmol/l (range 0.01-0.48 nmol/l) in premenopausal patients and 0.15 nmol/l (range 0.04-1.05 nmol/l) in postmenopausal patients. FSH serum concentrations correlated negatively to inhibin B levels (Spearman's rho -0.75,  $p < 0.0001$ ), demonstrating the suppression of pituitary FSH secretion by tumor derived inhibin B. Serum E2 levels above normal reference limits used in our hospital laboratory (2.38 nmol/l for premenopausal and 0.1 nmol/l for postmenopausal patients) were seen in 60% of AGCT patients, which is in line with previous data reporting estrogenic activity in approximately 70% of AGCTs (171, 177).

### 3.5 FSH increases E2 production in AGCT cells in vitro

To assess the functional effects of FSH in AGCTs, we stimulated AGCT cells with human recombinant FSH for 96 hours. Stimulation with FSH resulted in increased mRNA expression levels of *CYP19A1* in primary patient-derived AGCT cells and an increased secretion of E2 into the culture medium (Figure 16B). This was also seen in the KGN cell line. Also the FSH stimulated mRNA expression levels of all estrogen receptors (*ESR1*, *ESR2* and *GPER1*) were increased in AGCT cells. Interestingly, FSH stimulation increased cell number in three of six AGCT primary cell cultures by 26 to 43 %, however this was not seen in KGN cell line (Figure 16C).

Our results indicate active FSH signaling in AGCT cells, leading to increased estrogen production. We were also able to show that stimulation with FSH increases the viable cell number in AGCT cultures. On the other hand, AGCT seems to maintain its ability to restrict its own growth: by secreting inhibin B, it regulates tumor promoting signals by FSH. Furthermore, low FSH levels in AGCT patients support the idea of FSH independent growth of AGCTs. Although some studies and case reports have linked infertility treatments with gonadotropin stimulations to AGCTs, there is evidence showing that AGCT risk is not increased in this patient group (260). In conclusion, even though FSH may have a stimulatory effect on AGCT cells, it cannot be regarded as a pivotal driver of AGCT pathogenesis.

### 3.6 E2 increases AGCT cell viability only at high concentrations

Estradiol is considered to have mitogenic potential in many cancer subtypes and for example hormonal treatment in breast cancer relies mostly to estrogen inhibiting effects (261). However, in previously published studies with KGN cell line, estradiol has not been able to promote AGCT growth (175, 177). One study even reported diminished metastasizing properties in KGN cells after E2 stimulation (181), a result which was not confirmed by others (175).

For the first time, we studied the effects of E2 in primary AGCT cultures. We could not detect any significant change in cell viability after E2 stimulation at 10-100 nM concentrations (Figure 16A). We thus confirmed the earlier findings with KGN cell line. However, by using the highest concentration of E2 (1000 nM), increased cell number was seen in four out of the six studied tumor cell cultures, on average by 36 % (range 23-57%) (Figure 16A). High estradiol concentrations up to 1000 nM were detected from AGCT cell culture supernatants after FSH stimulation (Figure 16B), indicating that high local E2 concentrations can exist in AGCTs. In the clinical setting, these locally high E2 concentrations could appear for example during the perimenopause when circulating FSH levels are high, consistent with the typical onset of AGCTs. However, physiological circulating E2 levels do not seem to affect AGCT growth. This negligible response to E2 is also in line with our earlier results, in which postmenopausal hormone therapy was not

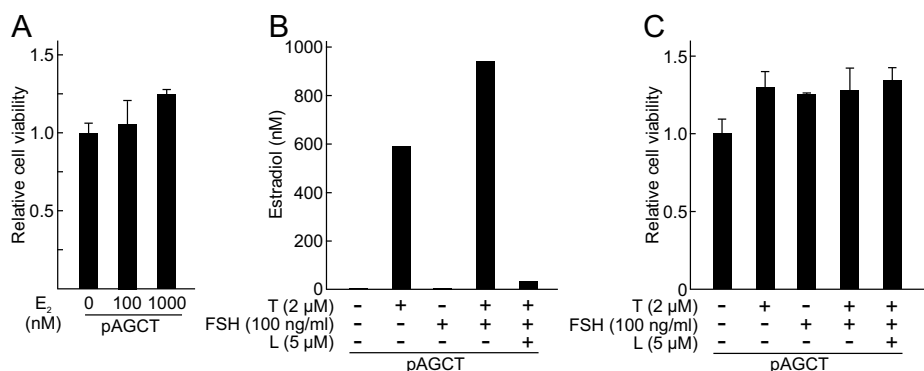
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shown to affect AGCT-related survival or tumor recurrence (8). This supports the idea that estrogen replacement therapy may be considered in AGCT patients with severe postmenopausal symptoms. Nevertheless, no randomized studies on this subject exist.

In other hormone-related malignancies, such as breast and prostate cancer, ER $\alpha$  is considered tumorigenic whereas ER $\beta$  mostly functions as a tumor suppressor by blocking the proliferation and inducing apoptosis (262-264). Considering that ER $\beta$  is the dominant estrogen receptor in AGCTs and considering that E2 had no significant stimulatory effect at physiological concentrations in AGCT cells, our results support a similar role in AGCTs. A recent study proposed that in addition to normal ER $\beta$ , AGCTs also express two different splice variants ER $\beta$ 2 and ER $\beta$ 5, of which ER $\beta$ 2 was capable of inhibiting AGCT apoptosis (265, 266). Here we only assessed the AGCT immunoreactivity to wild type ER $\beta$  and thus cannot evaluate the influence of splice variants to estrogenic actions. Increased ER $\beta$  expression in recurrent tumors suggests the significance of ER $\beta$  in this unique slow growing tumor type, but the specific effect of ER $\beta$  in tumor progression awaits further evidence.

### 3.7 Aromatase inhibition with letrozole suppresses E2 production in AGCT cells but does not affect cell viability

As CYP19A1 inhibitors have been used in AGCT treatment, we studied their effects in AGCT cell models. Cells were treated with CYP19A1 inhibitor letrozole and testosterone was provided as a substrate. Testosterone significantly stimulated E2 production in primary AGCT and KGN cells, and as expected, this effect was enhanced by FSH (Figure 16B). After letrozole treatment, E2 production in the cell cultures was completely suppressed, but no effect on cell viability in either primary AGCT or KGN cells was seen (Figure 16C). Surprisingly, testosterone stimulation increased the cell number in both AGCT and KGN cells.



**Figure 16.** A. Estradiol increased AGCT cell viability only at markedly high concentration (1000 nM). Cell viability was measured after stimulation with E<sub>2</sub> (0-1000 nM) for 96 hours. B. Letrozole blocked E<sub>2</sub> production in AGCT cells, E<sub>2</sub> concentrations were measured from cell culture supernatants after 96 hour treatment with letrozole. C. Testosterone increased the cell number in AGCTs. Even though letrozole inhibited E<sub>2</sub> production, it did not affect cell viability in AGCTs. All the columns depicted in A-C show responses of one representative tumor.

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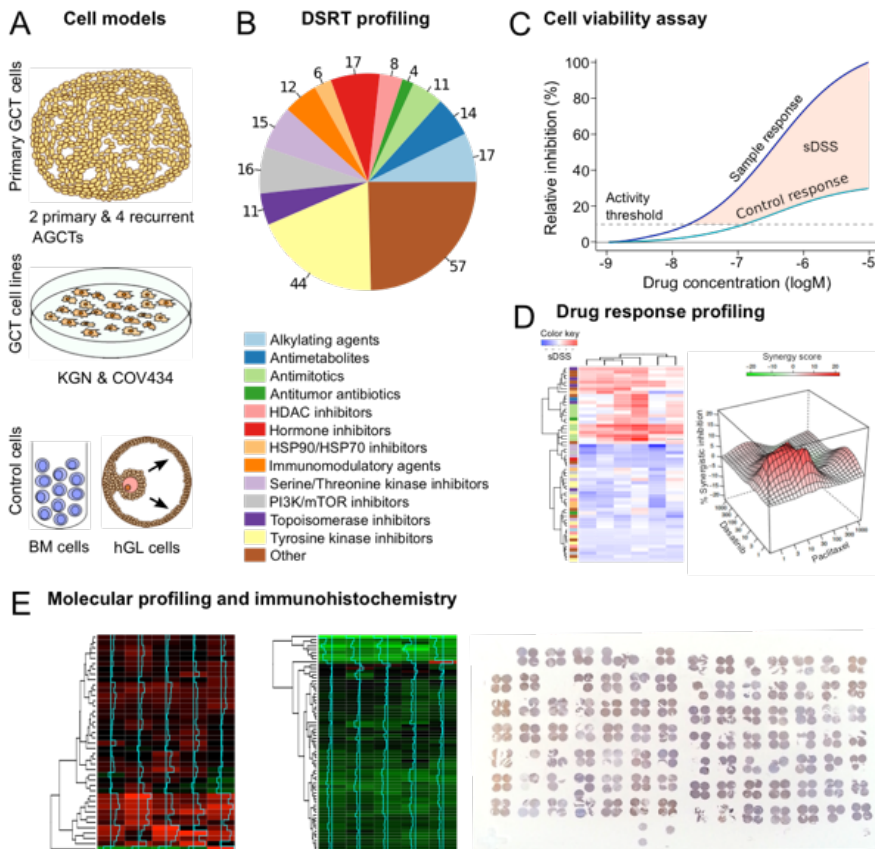
In conclusion, estradiol increased AGCT cell viability only at high concentrations. These concentrations are extremely higher than circulating E2 levels, but reflect the paracrine conditions in AGCT tissue. However, our results regarding negligible effect of letrozole in AGCT do not support the concept of aromatase inhibition in AGCT therapy. Surprisingly, we noted that the cell number was increased after testosterone stimulation, raising questions on the role of androgens in AGCT. This issue has not been addressed earlier on functional level. In normal granulosa cells there is contradictory data on the effects of testosterone on cell proliferation and apoptosis (267-269). Androgen receptors have been verified to be expressed in AGCTs implying to an androgenic reactivity of these cells (175, 179, 180).

In the current literature there are multiple case reports on positive responses to hormonal treatments and among these therapies aromatase inhibitors have been the most promising (66). Nevertheless, case reports are known to have a publication bias as negative responses are usually not reported. So far the only published study using objective RECIST criteria in AGCTs showed only modest efficacy for anti-hormone compounds: the response rate for all anti-hormone treatments was only 18%, of which none was seen after aromatase inhibitor (AI) treatment (73). However, seven out of ten patients using AIs had stable disease for up to 14 months of AI use. Considering the slow growth type of AGCT, stable disease may not be a response to a medication but the natural course of the disease. It is certainly possible, that the response would be better, if aromatase inhibitors would be used earlier in disease course.

Due to the complexity of hormonal homeostasis, hormonal effects are particularly challenging to study in cell models. Here we could only assess the direct effects of FSH and estradiol in AGCT cells, as imitating the *in vivo* hormonal regulation was not possible. This is a natural limitation of the study. Moreover, due to our small sample size, we cannot exclude that a subset of AGCT patients could gain benefit on aromatase inhibitors. It is also notable that these drugs are usually well tolerated and easily accessed. In light of our results, anti-hormone treatments suppressing both estradiol and testosterone concentrations could be more efficient in AGCT treatment. Nevertheless, more studies on androgen responsiveness of AGCTs are required. Finally, prospective clinical studies are needed to determine the effectiveness of hormonal therapies in AGCT.

#### 4. Drug sensitivity and resistance testing of AGCTs (IV)

Targeted therapies are usually discovered by studying the pathogenetic mechanisms of the disease in question, and thus identifying potential drug targets. To complement this approach, we used comprehensive screening of primary cultured AGCT cells with a large panel of oncologic compounds to identify novel potential drugs for treating AGCT. In addition, we combined gene expression analysis with high throughput drug screening of the studied tumors.



**Figure 17.** Schematic illustration of drug sensitivity and resistance testing combined with molecular profiling of the tumors. *A.* Cell models used in drug sensitivity and resistance testing (DSRT). *B.* Classification of used drugs. *C.* Responses were quantified by a drug sensitivity score (DSS). *D.* Responses were visualized in a heat map. *E.* Molecular fingerprints of tumors were analyzed by RNA seq and expression of selected drug targets was studied by immunostaining.

#### 4.1 AGCT profiles for drug responses and gene expression are consistent

In this study, we tested the drug responses using primary cell cultures derived from the tumors of seven AGCT patients: three primary and four recurrent tumors. Furthermore, we tested the responses of two GCT cell lines: KGN representing AGCT known to harbour the pathognomonic *FOXL2* 402C>G(C134W) mutation, and COV434, derived from a juvenile GCT that lacks the mutation and endogenous *FOXL2* expression. For controls, we used normal human granulosa luteal (hGL) cells collected and isolated from patients undergoing IVF treatment, and normal bone marrow mononuclear (BM) cells from healthy donors.

The drug panel consisted of 230 compounds including both traditional chemotherapeutics, approved targeted drugs and investigational regimens. We assayed the drug responses using a cell viability assay. To quantify the drug response profiles, we calculated the drug sensitivity score (DSS), which allowed the comparison of drug responses between different tumors and between different compounds (218). The mathematical algorithm for DSS has been developed specifically for drug sensitivity and resistance testing (DSRT) and it is considered more specific than commonly used IC50 values.

The DSS values between the AGCT samples were relatively consistent (Spearman's correlation  $r=0.55-0.79$ ), and the average AGCT response profile correlated highly with the KGN profile ( $r=0.83$ ). The correlations with the COV434 cell line and hGL were a little weaker ( $r=0.74$  and  $r=0.68$ , respectively). The gene expression profiles between the AGCT samples and the KGN cells were also highly concordant. The gene expression profiles of the AGCT samples correlated with each other ( $r=0.92-0.96$ ) while the average AGCT gene profile correlated with KGN ( $r=0.84$ ).

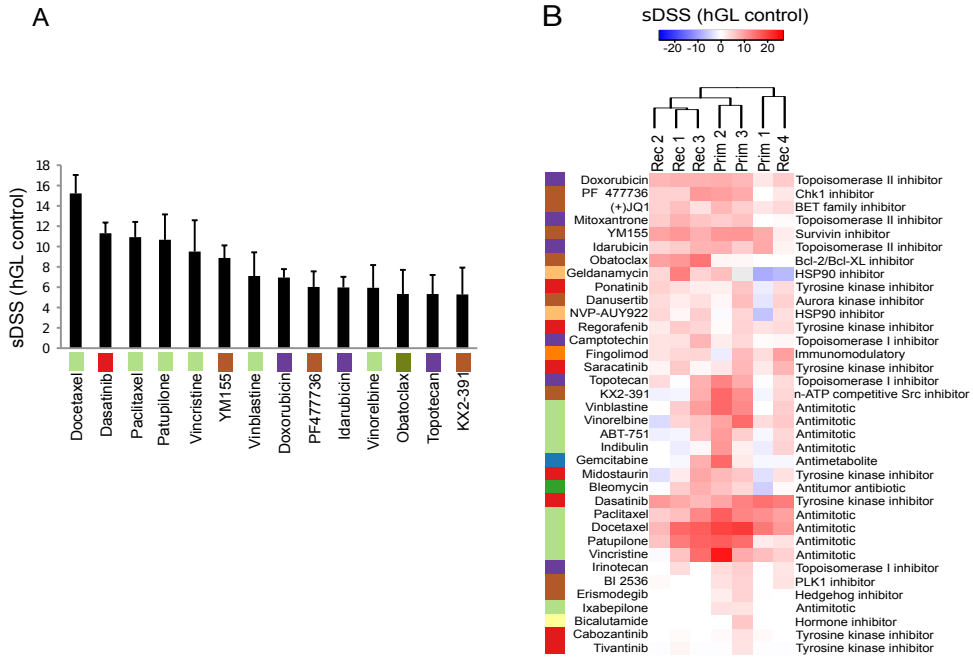
The similar drug responses between AGCTs were not surprising, as earlier studies have shown only limited molecular variation between individual tumors (44, 270). Common oncogenic driver mutations leading to formation of genetically diverse cell clones are not common in this tumor type. Also the relatively high similarity of drug response profile with the hGL cells was expected, as AGCTs have been shown to resemble normal proliferating granulosa cells of preovulatory follicles (reviewed in (271)). Our results also confirm the usefulness of KGN cells as a feasible cell model for AGCT when primary tumor cells are not available.

#### 4.2 AGCT cells exhibit selective sensitivity to tyrosine kinase inhibitor dasatinib

We next analysed the selectivity of drug responses in relation to control cell models. Selective drug sensitivity scores were first assessed by using hGL cells as a reference. Altogether 14 compounds were recognized as highly AGCT sensitive, with the mean sDSS value exceeding 5 in the studied tumors (Figure 18A). This group of sensitive drugs included several traditional chemotherapeutics such as docetaxel and paclitaxel. However, the most

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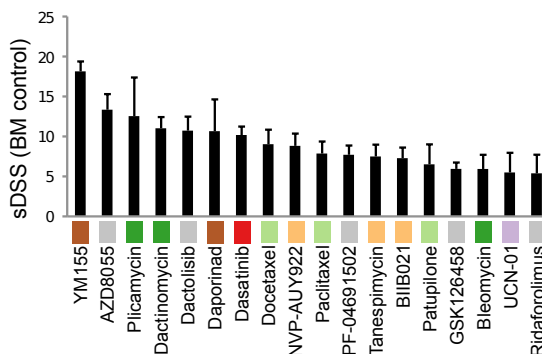
sensitive targeted compound was multi-tyrosine kinase inhibitor dasatinib (mean sDSS=11.3, range 7.4-15.0). Survivin inhibitor YM155 and CHK1-inhibitor PF477736 also showed selective response over the hGL cells (mean sDSS 8.5 and 5.5, respectively). In addition to all tested AGCT samples, also KGN cell line was sensitive to dasatinib (sDSS=16.2).



**Figure 18.** A. Average drug sensitivity score (DSS) values of seven AGCT samples were compared with the DSS values of human granulosa luteal (hGL) cells. Multiple traditional chemotherapeutics were represented. Multi-tyrosine kinase inhibitor dasatinib appeared as a most selective targeted compound. B. Drug responses of each AGCT sample were visualized in a heatmap plot. Red box exhibits high selectivity whereas blue box exhibits higher DSS value in the control sample.

Also, when selectivity was assessed against bone marrow mononuclear cells, dasatinib appeared selective with a mean sDSS of 10.2 (Figure 19). Survivin inhibitor YM155 exhibited the highest sensitivity in this comparison (sDSS 18.1). Multiple PI3K/mTOR inhibitors and heat shock protein 90 inhibitors were also represented among the selective regimen. Finally, the responses were evaluated against a collection of 68 cell lines from solid cancers (including breast, ovarian, lung and pancreatic cancers). Even in this comparison dasatinib and YM155 were among the most selective compounds, underlining their putative role in AGCT.

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**Figure 19.** Average drug sensitivity score (DSS) values of seven AGCT samples were compared with the DSS values of bone marrow mononuclear cells. Among selective compounds there were multiple mTOR inhibitors (grey box) and HSP90 inhibitors (orange box). Tyrosine kinase inhibitor dasatinib appeared among the selective compounds.

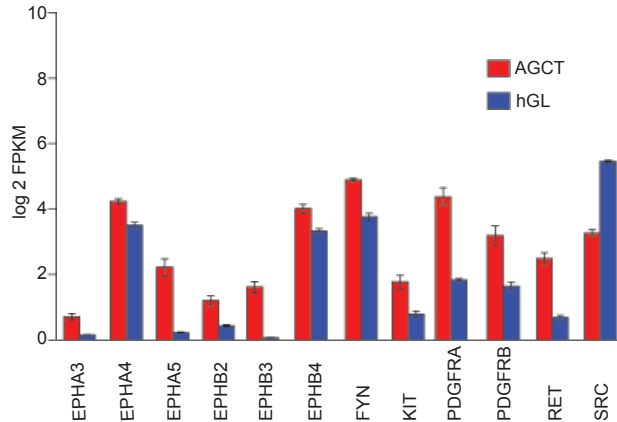
In the DSRT analysis control cells play a crucial role as the aim is to discover compounds that are selectively efficient in tumor cells. It is notable that when selectivity was assessed against hGL cells, several antimetabolic compounds and topoisomerase inhibitors showed efficacy. This is most likely due to the limited ability of hGL cells to divide *in vitro* compared to primary cultured AGCT cells. Certainly there are also limitations with the other control cell model used in the study (bone marrow mononuclear cells), as they are very distinct by their growth type and molecular background in comparison to AGCT cells. However, the different control cells including the collection of 68 cell lines complement each other and emphasize the selectivity of dasatinib in AGCTs.

### 4.3 Dasatinib targets are widely expressed in AGCTs

As dasatinib showed effectiveness in reducing AGCT cell viability, we aimed to examine the molecular background behind this response. Dasatinib has originally been developed for targeting ABL and SRC proteins, however it also inhibits a variety of other tyrosine kinases including PDGFR  $\alpha$  and  $\beta$ , c-Kit and ephrin receptors (reviewed in (272)). We performed total RNA sequencing of five AGCT samples, and did not find codon-changing or protein-truncating variants in dasatinib target genes. PDGFR  $\alpha$  and  $\beta$ , c-KIT, SRC family member FYN and some ephrin receptors (EPHA5 and EPHB3) were expressed at higher levels in AGCT compared to hGL cells. (Figure 20). High expressions for SRC, PDGFR and EPHA5 in AGCTs were also verified at protein level by immunostaining of the AGCT TMA.



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**Figure 20.** Several targets of dasatinib are expressed in AGCTs. The average mRNA expression levels of five AGCT samples are shown. The average mRNA expression of 3 pooled hGL cell samples were used as control. FPKM (fragments per kilobase of exon per million) values are represented in log2 scale. Red bars implicate the AGCT samples and blue bars hGL samples.

A case report describes a response to multi-tyrosine kinase inhibitor imatinib in recurrent AGCT (196). On this basis, effects of various tyrosine kinase inhibitors (TKIs) have earlier been studied in KGN cells (273, 274) resulting in negligible effects of compounds targeting primarily PDGFR and KIT. Also in our study, apart from dasatinib, tested PDGFR and KIT inhibitors were not effective, suggesting these kinases may not act as primary mediators of dasatinib response in AGCTs. Many SRC family members are widely expressed in AGCTs and we also noted response for another SRC inhibitor saracatinib in a subset of AGCTs (sDSS 5.7 and 7.8). Earlier, SRC has been shown to have a role in various endocrine cancers. It has been reported to promote progression of breast and prostate cancer and it has been associated to both estrogen and androgen receptor activation (275-278). Besides SRC family members, the role of ephrin receptors in AGCTs is of interest. The expression levels for several members of ephrin receptor family have been shown to be increased by FSH in normal granulosa cells, and ephrin signaling has been reported to affect morphology and adhesion of these cells (279). However, further studies are needed to examine the role of ephrin receptors in AGCTs.

Due to the selective efficacy of several mTOR inhibitors against the BM control, we also studied the mutation status and expression levels for the members of PI3K/mTOR signaling pathway. No clinically relevant mutations were detected and even the expression levels between AGCTs and hGL cells were very similar. Immunostaining confirmed high expression of PI3K and mTOR in both AGCTs and normal granulosa luteal cells. The PI3K/Akt/mTOR pathway has proven crucial in promoting growth and differentiation of granulosa cells, and our results showing robust expression of pathway components support this in both healthy and malignant granulosa cells. It is notable that the hGL cells were

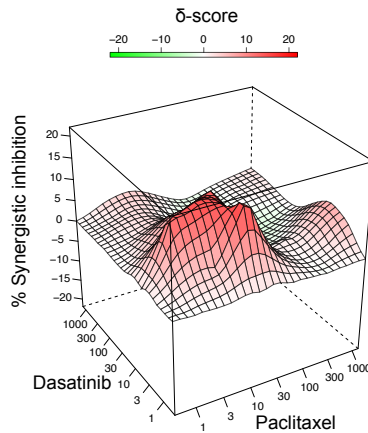
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collected from patients undergoing IVF treatment and are thus hormonally primed. As PI3K/mTOR pathway is known to be regulated by FSH, it is possible that the high mRNA expressions seen in hGL cells are partly due to stimulating effect of FSH. In a previous study activation of PI3K/mTOR signaling was reported in AGCTs (280, 281) and there is also earlier evidence on mTOR inhibition in reduction of AGCT growth (282). Our results support this evidence for mTOR inhibition in AGCTs.

Except for the known *FOXL2* (C134W) mutation present in all tested samples, RNA-sequencing did not reveal mutations in either AGCT associated genes or in known oncogenes. This is in line with earlier studies reporting a low level of mutations in AGCTs (44, 270).

### 4.4 Dasatinib or PI3K/mTOR inhibitors exhibit synergy when combined with paclitaxel

As dasatinib was selective against all tested control cells, we evaluated its efficacy in combination with paclitaxel, a traditional chemotherapeutic. For this experiment, we used both KGN and COV434 cell lines. Interestingly, combination of dasatinib and paclitaxel resulted in synergistic inhibition of cell viability in the *FOXL2* mutation positive KGN cells (Figure 21) but in *FOXL2*-negative COV434 cells this was not seen. The synergistic effect was detected already at low concentrations: 1-10 nM for dasatinib and 3-30 nM for paclitaxel. In addition to dasatinib, also mTOR inhibitor everolimus showed synergy in combination with paclitaxel at relatively low levels.



**Figure 21.** Synergistic effect of dasatinib and paclitaxel in KGN cell line. The color is scaled in red (synergistic,  $\delta$ -score  $> 1$ ), white (50% inhibition) and green antagonistic,  $\delta$ -score  $< 1$ ).

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In this unbiased drug screen, we identified an approved oncologic drug as effective in reducing AGCT cell viability. Dasatinib is currently used in the treatment of chronic myeloid leukemia, where its efficacy is mostly conveyed through inhibition of the BCR-ABL oncoprotein (283). However, dasatinib also targets a variety of other kinases and multiple clinical trials are currently evaluating its efficacy in solid cancers. Hitherto, successful responses for dasatinib in combination with chemotherapeutics have been reported for example in prostate and ovarian cancers (284, 285). In previous *in vitro* studies, dasatinib was shown to enhance the antitumor efficacy of paclitaxel by down-regulating Src phosphorylation (286). The adverse side-effects related to dasatinib have appeared tolerable, although infrequent complications such as pulmonary hypertension have been restricting its use in some patients (287). It is notable, that synergistic activity in combination treatments enables the use of lower drug concentrations than in single treatments. Combination of dasatinib and paclitaxel seems thus clinically feasible and is suggested for further clinical testing in AGCTs.

Survivin inhibitor YM155 showed also high selective efficacy on AGCTs. Survivin is expressed in a multitude of cancers and is also described as antiapoptotic molecule in normal granulosa cells (148, 149). In KGN cells, survivin inhibition was recently shown to enhance apoptosis induced by TRAIL (288). Unfortunately, the clinical efficacy of YM155 is poor and for the present no approved survivin inhibitors exist. However, survivin has lately been considered as a target for immunotherapy, and is currently evaluated as an antigen in vaccine-induced anti-cancer immune responses (289).

In addition to these potentially effective drugs, several compounds showed no efficacy in the drug screen. This was notable concerning the anti-hormone compounds, including the aromatase inhibitors, which are currently used in AGCT treatment. Also compounds targeting epidermal growth factor, earlier implied as possible AGCT therapeutics, showed no efficacy in this screen.

### 4.5 DSRT as a useful tool in drug repurposing

The DSRT approach was originally utilized in hematological malignancies, showing diverse responses among individual patients which were related to combined with distinct genomic features in the tested samples (215). Now we applied the approach to a very distinct solid tumor type exhibiting only low intertumoral and intratumoral variation. Considering the growth type and genomic stability of AGCTs, the consistency between drug responses was not surprising, even though our samples included both primary and recurrent tumors.

A crucial obstacle in developing AGCT treatments is the rarity of the tumor, which limits opportunities for clinical trials. Recruitment of trial participants is prolonged and considering the slow growth pattern of tumor, follow-up times in trials should be long enough to assess the efficacies of the tested regimen. Another recognized problem with AGCT is the lack of suitable *in vivo* animal models. Several transgenic mouse models have

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been generated, including knockouts for inhibin  $\alpha$  -gene and Smad1/5s (290, 291). Nevertheless, all these mouse models have had serious limitations and above all, none of them has exhibited the mutated *Foxl2*. This lack of appropriate animal model further highlights the significance of other preclinical approaches to test new therapies for AGCTs. By using high throughput screening methods, putatively effective compounds can be identified and selected to further clinical testing. This method is especially valuable in rare tumors like AGCT.

## Conclusions and future prospects

This study aimed to develop the treatment of AGCTs by utilizing a vast collection of tissue and serum samples from AGCT patients with an extensive follow-up data. As the efficacy of the present chemotherapy in AGCT is only modest, new treatments for advanced and recurrent disease are urgently needed. Due to the rarity of AGCT, randomized clinical trials are challenging to organize. This underlines the significance of preclinical studies in evaluating compounds that could be further clinically tested.

Optimally performed surgery is crucial for the prognosis, and thus AGCTs should be recognized preoperatively. Our results show that commonly used epithelial serum marker HE4 has no role in AGCT diagnostics and emphasize that normal CA125 and HE4 levels do not rule out a malignant ovarian tumor. Thus, in case of unknown ovarian masses especially with cystic-solid appearance and clinical symptoms potentially linked to AGCT, measuring inhibin B is strongly recommended. In premenopausal patients, combined inhibin B and AMH measurement is beneficial.

In this study we found out that TRAIL tissue expression was reduced in large AGCTs. Furthermore, TRAIL levels in serum samples of these patients correlated negatively with tumor size, as patients with larger tumors had lower serum TRAIL levels. Considering the previous studies showing the ability of TRAIL to induce apoptosis in AGCT cells, our present findings support further studies on TRAIL as a targeted treatment for AGCTs. Interestingly, in a recent study inhibition of survivin, an anti-apoptotic protein, was shown to enhance the efficacy of TRAIL in KGN cells (288). Also in our high throughput drug screening of primary AGCT cells, survivin inhibitor YM155 selectively inhibited AGCT cell viability. These results encourage future studies of combination of TRAIL and other apoptosis enhancing compounds including survivin inhibition in AGCT treatment.

The role of hormonal factors in AGCT pathogenesis is intriguing. We characterized the hormonal milieu of AGCTs, yet more research is needed to define the exact roles of various hormone receptors in AGCT. This was the first time when FSH was shown to increase AGCT cell viability. We also reported the negligible effect of E2 stimulation in AGCT cells. We conclude that FSH seems to contribute to AGCT growth, however it does not seem to be the crucial driver in AGCT pathogenesis. AGCTs seem to maintain self-regulatory mechanisms even in recurrent tumors, including production of inhibin B and AMH by the tumor cells. This may in part be responsible for the indolent growth of the tumor. Based on our results, aromatase inhibitors do not inhibit AGCT cell growth. Due to our small sample size, we cannot exclude that some patients may benefit from aromatase inhibitors or other hormonal treatments. Further studies should assess the functional pathways of androgen action in AGCTs and study whether inhibiting both estrogen and androgen signalling would be more beneficial in AGCT treatment. Moreover, predictive biomarkers allowing the selection of patients gaining benefit from the hormonal treatments should be recognized.

## CONCLUSIONS AND FUTURE PROSPECTS

Based on drug sensitivity and resistance testing, we identified tyrosine kinase inhibitor dasatinib as a potential AGCT therapy. Sensitivity to dasatinib was observed in all tested AGCT samples, supporting its use in both primary and recurrent tumors. Interestingly, dasatinib showed synergy effect when combined with paclitaxel. We detected no mutations in dasatinib target genes, though most of these molecular targets were highly expressed in AGCTs. Future studies are needed to recognize the mechanisms behind dasatinib response and especially the roles of Src family proteins and ephrin receptors need further investigation. DSRT offers a useful platform to study drug responses in rare tumors like AGCT, however future studies should place more effort to develop a more advanced cell model for the drug testing. 3D-cell cultures would be more representative considering the effects of tumor microenvironment to drug responses. Also, it would be interesting to have an opportunity to test subsequent tumor samples from serial recurrences from a single patient. This could provide information on tumor evolution during the course of the disease. Nevertheless, the herein observed synergy between dasatinib and paclitaxel encourages clinical testing of this combination in AGCT patients. Considering the rarity of the tumor, international collaboration is crucial to ensure adequate numbers of patients in future clinical trials.

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